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EDUCATIONAL BOOK

Editor
Anthony R. Green (Cambridge)
Chairman of the Educational Programme
EHA-5 Educational Book, June 2000

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Foreword

This book contains manuscripts corresponding to presentations in the Educational Programme of EHA-5. We hope that the contents will be of interest to both clinical and experimental haematologists. Each session was designed to provide concise and informative updates on significant developments in specific areas of haematological science.

The book is published by the European Haematology Association. It has been produced through the efforts of many people. We thank particularly Mr. Michele Moscato and Ms. Sara Harrop for all their hard work together with Professor Victor Hoffbrand, Dr. Brian Huntly and Dr. Adrian Bloor for their generous help with editing. We would also like to express our gratitude to all the authors and chairmen for their expert contributions to the 2000 programme.

We are sure you will find the book valuable and enjoyable.

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Indolent lymphomas. Different entities and diagnostic problems

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Small B-cell lymphomas, which usually have an indolent course, are a very heterogeneous group of clinicopathological entities. They have a broad morphological spectrum and their diagnosis may be difficult.

In the last 15 years, new immunological, cytogenetic and molecular knowledge has resulted in the refinement of their classification and the definition of well-defined entities, described in the last international classifications of lymphomas.

The Revised European-American Classification of Lymphoid Neoplasms (REAL classification) proposed by the International Study Group in 1994 is based on a significant degree on the updated Kiel classification; the forthcoming WHO classification will be derived from the REAL classification.

In these classifications the distinct entities can be easily recognised by pathologists and have clinical relevance. They are characterised by morphological, immunophenotypical, and genetic features in combination with their clinical presentation and natural history, and to the extent possible, according to their presumed normal cell counterpart. Variations in grade are possible within a disease as well as morphological variants and clinical subtypes (specific clinical presentation or course).

The equivalents for each small B-cell lymphoma entity in the Working-Formulation, updated Kiel, REAL and forthcoming WHO classifications are summarised in Table 1.

The normal cell counterpart of small B-cell lymphomas

These lymphomas are among the best examples of how malignant lymphomas can be related to the immune system. They are considered the neoplastic equivalents of normal B-cell compartments, which are summarised in Figure 1.

Chronic lymphocytic leukemia/ small lymphocytic lymphoma (B-CLL/ B-SLL)

The leukaemic form B-CLL comprises 90% of chronic lymphoid leukemias in Western countries. It occurs in older adults, and involves bone marrow, peripheral blood, nodes, spleen, and liver; it has an indolent course. A non-leukaemic nodal presentation, corresponding to B-SLL, is less frequent. Histologically, these neoplasms are composed of small lymphocytes with clumped chromatin, and clusters of larger cells, the prolymphocytes and para-immunoblasts (pseudofollicles or proliferation centres).

In some cases the nuclei are irregular (leading to a differential diagnosis with mantle cell lymphoma). A variant with plasmacytoid differentiation (sometimes with monoclonal gammopathy) may have a poorer prognosis and corresponds to cases considered in the Kiel classification as lymphoplasmacytoid immunocytoma. Interfollicular B-PLL grows between and around reactive follicles, sometimes with a mantle or marginal-zone pattern, it is difficult to diagnose.

The demonstration of the classical immunophenotype (faint sIgM + IgD+/– panB markers + CD5+ CD23+ CD43+ FMC7–) is very useful in cases with atypical morphology.

These disease are characterized by rearrangement of Ig genes, usually without somatic mutations (prefollicular B-cells). But a subset of B-CLL shows isotype switching and static somatic mutations, and probably the physiopathology of B-CLL/B-SLL is complex.

Cytogenetic abnormalities are inconstant, including trisomy 12, 13q14 deletions and translocations, 11q deletions and 17p deletions/translocations (associated with a poorer prognosis).

Tumoral progression occurs either with increased prolymphocytes (10-50%) in the peripheral blood (mixed type of B-CLL defined by the FAB group) or, in a minority of cases, transformation to high grade lymphoma (Richter’s syndrome), usually large B-cell lymphoma, sometimes Hodgkin’s disease.

The postulated normal counterpart is recirculating naïve CD5+ CD23+ peripheral B cell

Prolymphocytic leukaemia (B-PLL) is rare, and considered as a distinct entity in the WHO classification. Clinical presentation is different with a high white blood cell count and a prominent splenomegaly, and has a more aggressive clinical course. The phenotype is different (CD5+/CD23+ FMC7+) and the normal cell counterpart is unknown. B-PLL is probably a heterogeneous disease, not corresponding to a single entity, some cases being prolymphocytic progression of B-CLL and others progression of other types of small B-cell lymphomas: mantle cell lymphoma (MCL) or marginal zone B-cell lymphoma (MZL).

Lymphoplasmacytoid lymphoma/ immunocytoma

This rare indolent disease (1-2% of nodal lymphomas) occurs in older adults. Sites involved include bone marrow, lymph nodes, spleen, and sometimes
peripheral blood. LPL is often associated with Waldenström’s macroglobulinaemia (monoclonal IgM paraprotein in serum, with hyperviscosity symptoms), or H9253 heavy chain disease, autoimmune haemolytic anaemia or cryoglobulinaemia. Recently, association with hepatitis C infection and type II mixed cryoglobulinaemia has been shown by some Italian researchers.8

Histologically, the pattern is diffuse without pseudofollicles, sometimes interfollicular, with sparing of the sinuses. A few reactive follicles may be present. The tumoral cells are small lymphocytes, lymphoplasmacytoid lymphocytes (with abundant basophilic cytoplasm) and plasma cells, with or without Dutcher bodies (PAS+ intranuclear inclusions). Rare immunoblasts are often present. Admixed epithelioid cells and mast cells may be seen.

The phenotype (strong surface and cytoplasmic Ig usually IgM, rarely IgA, IgD+, panB markers+, CD5-/CD10- CD23+ CD43+) allows the distinction from other small B-cell lymphomas (B-CLL/SLL or follicular lymphoma) which can, in rare cases, show a plasmacytic maturation. The term LPL/immunocytoma is restricted to tumours lacking features of these other lymphomas, but there is a quite complete overlap (morphological and immunological) with marginal zone B-cell lymphoma characterised by frequent plasmacytoid/cytic differentiation.9 Most cases previously diagnosed as immunocytoma in extranodal sites are examples of MALT lymphomas and possibly in the future, LPL and MZL will be considered as a single entity.

Genetic features include rearrangement of Ig genes and somatic mutations (post-follicular B-cells). The t(9;14) (p13;q32) translocation between the B-cell specific transcription factor PAX-5 and the Ig heavy chain locus has been reported in LPL.

Transformation can occur to diffuse large B-cell lymphoma (usually immunoblastic). Intermediate features with an increase in the number of immunoblasts and mitoses (included in the polymorphic immunocytoma of the Kiel classification) probably imply a worse prognosis than typical histology, but validated morphological criteria for grading have not been established and other biological markers of tumoral progression are probably useful.

The postulated normal counterpart is a CD5 negative peripheral B-cell maturing to a plasma cell.

<table>
<thead>
<tr>
<th>Working Formulation</th>
<th>Updated Kiel</th>
<th>REAL</th>
<th>WHO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small lymphocytic, consistent with CLL</td>
<td>B-lymphocytic, B-CLL</td>
<td>B-cell chronic lymphocytic leukaemia</td>
<td>B-CLL/small lymphocytic lymphoma variant; with plasmacytoid differentiation subtypes:</td>
</tr>
<tr>
<td>Small lymphocytic, plasmacytoid</td>
<td>prolymphocytic leukaemia, immunocytoma, lymphoplasmacytoid</td>
<td>prolymphocytic leukaemia/lymphocytic lymphoma</td>
<td>with monoclonal gammopathy</td>
</tr>
<tr>
<td></td>
<td>Diffuse, mixed small and large cell</td>
<td>Lymphoplasmacytoid lymphoma/immunocytoma subtypes:</td>
<td></td>
</tr>
<tr>
<td>Small lymphocytic, plasmacytoid</td>
<td>Lymphoplasmacytoid</td>
<td>Lymphoplasmacytoid lymphoma/immunocytoma</td>
<td></td>
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<tr>
<td>Diffuse, mixed small and large cell</td>
<td>Diffuse, large cleaved cell</td>
<td>Mantle cell lymphoma</td>
<td></td>
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<tr>
<td>Small lymphocytic</td>
<td>Centrocyclic</td>
<td>Mantle cell lymphoma</td>
<td></td>
</tr>
<tr>
<td>Diffuse, small cleaved cell</td>
<td>Centroblastic, centrocytic</td>
<td>Mantle cell lymphoma</td>
<td></td>
</tr>
<tr>
<td>Diffuse, mixed small and large cell</td>
<td>Diffuse, large cleaved cell</td>
<td>Mantle cell lymphoma</td>
<td></td>
</tr>
<tr>
<td>Diffuse, small cleaved cell</td>
<td>Extramedullary marginal zone</td>
<td>Extranodal marginal zone B-cell lymphoma subtypes:</td>
<td></td>
</tr>
<tr>
<td>Diffuse, mixed small and large cell</td>
<td>(low grade B-cell lymphoma)</td>
<td>B-cell lymphoma</td>
<td></td>
</tr>
<tr>
<td>Small lymphocytic</td>
<td>Monocytoid, including marginal zone</td>
<td>Nodal marginal zone B-cell lymphoma (provisional)</td>
<td></td>
</tr>
<tr>
<td>Diffuse, mixed small and large cell</td>
<td>Immunocytoma</td>
<td>Nodal marginal zone lymphoma</td>
<td></td>
</tr>
<tr>
<td>Inclassifiable</td>
<td>Splenic marginal zone B-cell</td>
<td>Splenic marginal zone B-cell lymphoma (provisional)</td>
<td></td>
</tr>
<tr>
<td>Small lymphocytic</td>
<td>Hairy cell leukaemia</td>
<td>Splenic marginal zone lymphoma</td>
<td></td>
</tr>
<tr>
<td>Diffuse, small cleaved cell</td>
<td>Immunocytoma</td>
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<tr>
<td>Splenic marginal zone B-cell</td>
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<tr>
<td>Hairy cell leukaemia</td>
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Table 1. Comparison of 4 classifications of small B-cell lymphomas: Working Formulation (1982), updated Kiel (1992), REAL (1994) and forthcoming WHO.

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Mantle cell lymphoma (MCL)

This entity, which represents 5-6% of nodal lymphomas, has been progressively identified in recent years through the demonstration of its unique morphological, immunophenotypic, cytogenetic and molecular features. It was initially described as centrocytic lymphoma in the Kiel classification and as intermediate lymphocytic lymphoma in the modified Rapaport classification.

MCL is a disease of the elderly, occurring predominantly in males. Most patients present with a widespread disease (stage III or IV): generalised lymphadenopathy, involvement of bone marrow, spleen, blood, and Waldeyer's ring. Extranodal localisations are not rare, particularly in the gastrointestinal tract, sometimes with a peculiar multiple involvement (lymphomatous polyposis).

The course is moderately aggressive and the disease is incurable with currently available treatment. The median survival is 2-5 years; MCL is not an indolent lymphoma (lymphoma of intermediate grade).

Histologically, the lymph nodes show architectural effacement, with a vaguely nodular or diffuse pattern. Mantle zone and follicular patterns are rare. Residual naked germinal centers may be present. Pseudofollicular growth centres are absent.

In most cases (typical MCL), there is a very monotonous cytologic composition: the cells are small to medium-sized with indented nuclei, moderately dispersed chromatin, inconspicuous nucleoli and scant cytoplasm, without plasmacytoid differentiation. The mitotic rate is variable, usually low but sometimes high (this difference having a possible prognostic value). Large tumoral cells (centroblasts or immunoblasts) are absent or very rare, in contrast to the situation of follicular lymphoma. Follicular dendritic cells (FDC) are present, ranged in a loose or sometimes tight meshwork.

Three cytologic variants can be recognised (grouped under the term of blastoid variants):  
- **blastic variant**: cells resembling lymphoblasts, with a fine dispersed chromatin with high proliferative and apoptotic rates and frequently a starry sky pattern.
- **pleomorphic variant**: medium to large-sized cells are predominant, with a diffuse or nodular pattern. Large cells with cleaved nuclei resemble large centrocytes. These cases were classified as anaplastic centrocytic or centrocytoid centroblastic lymphomas in the Kiel classification.
- **small cell variant**: small to medium-sized cells with round nuclei and more clumped chromatin are predominant. Plasmacytoid differentiation is very rare but a monocytoid appearance, with clear cytoplasm is possible.

Several morphological aspects in the same patient may be observed in different sites, and indeed sometimes in the same lesion. During the evolution, histological transformation from a typical small cell to a blastic or large cell variant is rare. Usually, the morphology remains identical or there is an increase in mitotic activity.

The classical immunophenotype is sIgM+ (strong expression), usually IgD+, λ > κ, pan-B markers, CD5+, CD10+, CD23+, usually CD43+, CD11c+, bcl2 protein+, cyclin D1+. An atypical immunophenotype is possible (CD5- or CD23-). In these cases, typical morphology and the demonstration of overexpression of cyclin D1 are required. An expression of the mucosal homing receptor integrin 4β7 is observed in
cases with gastrointestinal dissemination.

Genetic features are rearrangement of Ig genes and no or very few mutations of variable regions. The t(11;14) (q13;q32) involving the Ig heavy chain locus and the bcl-1 locus in 11q13 is present in the majority of cases and represents a useful, although not specific, genetic marker. This translocation results in an overexpression of cyclin D1, a cell-cycle protein that is not expressed in normal lymphoid cells. This genetic event is thought to have an important role in the pathogenesis of MCL, possibly deregulating the normal cell cycle.

Its rate of detection depends on the method used, being found in 35% to 70% of cases using PCR, Southern blot analysis, and cytogenetics, but in almost all cases using DNA fibre FISH analysis. At RNA and protein levels, at least 90% of MCL show overexpression of cyclin D1 (Northern blot, RT-PCR, Western blot, slot-blot, immunohistochemistry), accompanied by downregulation of cyclin D3.

Cyclin D1 overexpression is rare in other B-cell lymphomas, although possible in plasmacytoma/myeloma and hairy cell leukaemia.

Additional chromosome abnormalities can be present, especially those involving chromosomes 1 and 12, loss of chromosome 6 or various chromosomal translocations. BCL2 and c-myc rearrangements are absent.

Mutations with overexpression of p53 have been demonstrated in a minority of MCL, as well as deletions and loss of expression of p16, p18 and p21 genes. These anomalies are associated with pleomorphic or blastic variants, which also harbour translocations. BCL2 and c-myc rearrangements are absent.

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The grading of FL, useful but difficult, is based on the number of centroblasts, but interobserver reproducibility is poor. Only 2 grades can be defined by the natural history and response to treatment. However, the classical 3 grade-system will be maintained in the WHO classification, because changing the nomenclature would be potentially confusing. The Berard cell-counting method is recommended: grade 1: 0-5 centroblasts/high power field (hpf); grade 2: 6-15 centroblasts/hpf; grade 3: >15 centroblasts/hpf.

The presence of diffuse large B-cell lymphoma in any follicular lymphoma indicates transformation to aggressive disease and should be reported as a separate diagnosis: e.g. FL grade 2, with diffuse large B-cell lymphoma. Some transformed cases have the morphology of Burkitt-like or B-lymphoblastic lymphoma.

Factors other than histological grade have prognostic value, for example clinical features (International Prognostic Index) and probably biological markers (BCL2 expression, p53 mutations ...).

The immunophenotype is: sIg+ (M,G,rarely A) pan B markers+ CD10+ CD5− CD43− CD23− bcl-2 protein−, bcl2 protein+ (also expressed in other low grade B-cell lymphomas, but not in reactive follicles).

Genetic features include rearrangement of Ig genes, extensive and ongoing mutations of variable regions, with evidence of antigen selection (follicle centre cells). The t(14;18) (q32;q21), present in 70-95% of cases, juxtaposes the BCL2-gene to the Ig heavy chain gene resulting in overexpression of the anti-apoptotic bcl2 protein. This translocation occurs early in B-cell development and other unknown genetic events are necessary for development of lymphoma.

Follicular lymphoma

In Western countries, this comprises about 35% of adult non-Hodgkin’s lymphomas. Most patient have widespread disease at diagnosis, involving lymph nodes, spleen, bone marrow, and rarely extranodal sites. The course is very indolent (median survival 7-9 years) but incurable, and progression to diffuse large B-cell lymphoma is frequent (occurring in up to 60% of cases).

Morphological diagnosis is usually easy because of the follicular (nodular) growth pattern. Diffuse areas may be present, often with sclerosis, and must be reported and quantified: predominantly follicular (>75% follicular), follicular and diffuse (25-75% follicular), predominantly diffuse (<25% follicular).

Most cases have two types of cells: small-to-medium cells with cleaved nuclei and scant cytoplasm (centrocytes/cleaved follicle centre cells) and large transformed cells with round nuclei, peripheral nucleoli and basophilic cytoplasm (centroblasts/non-cleaved follicle centre cells). Small cleaved cells usually predominate. Some cases show increased centroblasts, with a mixed cell composition. Rare cases have predominantly centroblasts. Plasmacytic differentiation, sometimes with signet ring cells, is very rare; a marginal zone pattern, with monocytoid B cells, is possible.

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Grades 1 and 2 are more closely related to each other than grade 3 (with earlier relapses, but same overall survival).

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The grading of FL, useful but difficult, is based on the number of centroblasts, but interobserver reproducibility is poor. Only 2 grades can be defined by the natural history and response to treatment. However, the classical 3 grade-system will be maintained in the WHO classification, because changing the nomenclature would be potentially confusing. The Berard cell-counting method is recommended: grade 1: 0-5 centroblasts/high power field (hpf); grade 2: 6-15 centroblasts/hpf; grade 3: >15 centroblasts/hpf.

Grades 1 and 2 are more closely related to each other than grade 3 (with earlier relapses, but same overall survival).

The presence of diffuse large B-cell lymphoma in any follicular lymphoma indicates transformation to aggressive disease and should be reported as a separate diagnosis: e.g. FL grade 2, with diffuse large B-cell lymphoma. Some transformed cases have the morphology of Burkitt-like or B-lymphoblastic lymphoma.

Factors other than histological grade have prognostic value, for example clinical features (International Prognostic Index) and probably biological markers (BCL2 expression, p53 mutations ...).

The immunophenotype is: sIg+ (M,G,rarely A) pan B markers+ CD10+ CD5− CD43− CD23− bcl-2 protein−, bcl2 protein+ (also expressed in other low grade B-cell lymphomas, but not in reactive follicles).

Genetic features include rearrangement of Ig genes, extensive and ongoing mutations of variable regions, with evidence of antigen selection (follicle centre cells). The t(14;18) (q32;q21), present in 70-95% of cases, juxtaposes the BCL2-gene to the Ig heavy chain gene resulting in overexpression of the anti-apoptotic bcl2 protein. This translocation occurs early in B-cell development and other unknown genetic events are necessary for development of lymphoma.

p53 mutations, p16 hypermethylation and c-myc rearrangement or overexpression have been reported in cases with histological transformation and aggressiveness.

The differential diagnoses are essentially follicular hyperplasia, and follicular colonisation by other small B-cell lymphomas (MZL, MCL, rarely interfollicular SLL). Immunophenotypic and genotypic studies are useful in these difficult cases.

One subtype and one variant of FL are described: • cutaneous primary FL, probably very rare, often
Marginal zone B-cell lymphomas

Marginal zone B-cell lymphomas (MZL) have gradually been identified over the last 15 years: first the extranodal localisations (mucosa associated lymphoid tissue, MALT-type) by Isaacson and Wright, then the primary nodal and splenic subtypes. Although some controversy remains concerning their relationship, the three subtypes will be officially accepted in the forthcoming WHO classification (nodal and splenic MZL were provisional in the REAL classification).

MZL lymphomas represent 7% of all lymphomas, whereas the frequencies of nodal and splenic MZL (2-3% of lymphomas) are probably underestimated. Historically, the 3 subtypes have certain common cytological and architectural characteristics.

Cytological features are very heterogeneous, with several cell types usually associated in varying proportions: small cells with irregular nuclei (centrocyte-like cells), more frequent in MALT lymphomas; small cells with more regular nuclei and clear cytoplasm (monocytoid B-cells), cells resembling small lymphocytes; small cells with plasma cell differentiation (marginal zone plasma cells, variable content of medium-to-large cells (centroblast or immunoblast-like). Follicular dendritic cells, usually ranged in a nodular meshwork, are always present. It is worthy of note that cases with plasmacytic differentiation were classified in the past as immunocytomas, which probably belong to the same entity.

The infiltration initiates in the marginal zone to extend to interfollicular areas or follicles, and several architectural patterns are observed: marginal-zone pattern (perifollicular or inverse follicular), interfollicular, perisinusoidal (in lymph nodes), follicular by colonisation of reactive follicles, which are common. A diffuse pattern is rare.

In MALT lymphomas, the tumoral cells invade and destroy the epithelial structures, forming so-called lymphoepithelial lesions. In splenic MZL (SMZL), there is a micronodular pattern (involvement of white pulp and red pulp), associated with a diffuse invasion of the sinuses. The typical white pulp aspect is biphasic, with central small cells surrounded by medium clear cells and a variable number of large cells. An exclusive diffuse infiltration of congestive red pulp by normal or atrophic white pulp is mostly observed in splenic MZL with villous lymphocytes (SVL), defined by the presence in peripheral blood, of at least 20% of typical villous lymphocytes showing clumped chromatin and basophilic cytoplasm with polar projections.

The typical phenotype (cd20+/− panB markers+/− CD5− CD10− CD23− CD43+/− bc12+/− cyclinD1+) is an important diagnostic feature with other small B-cell lymphomas (MCL, FL, plasmacytoid SLL). An atypical phenotype (CD5+ or CD23+ sometimes cyclinD1+−) is possible, with some borderline cases essentially with MCL.

Genetic features are Ig gene rearrangement and somatic mutations (post-follicular B-cells), but ongoing somatic mutations are extensive only in MALT lymphomas and less common in nodal and splenic MZL, indicating that perhaps these tumours originate from different subsets of marginal B-cells or have different mechanisms of lymphoma triggering. BCL2 and BCL6 are germline, the rearrangement of BCL1 has been described in some cases.

The most frequent cytogenetic abnormalities include trisomy 3 (60%), trisomy 18 (36%) and structural Ig abnormalities. They have been reported in all 3 subtypes of MZL. Others are specific to certain subtypes: del(3) and del(7q) are seen in SMZL. The t(11;18)(q21;q21) associated with low-grade MALT lymphoma juxtaposes the apoptosis inhibitor gene API2 and the MLT gene, and has probably an important role in early stages of tumorigenesis. The t(1;14) (p22;q32) implying the tumour suppressor gene BCL10 is observed in aggressive MALT lymphomas. The transformation of MZL to large B-cell lymphomas has also been associated with p53 inactivation, deletions of p16, sometimes the t(8;14) in MALT-type and del 7q32 in SMZL.

Other characteristics of each subtype of MZL will now be considered.

Extranodal MZL, of MALT type. Occurring in adults, predominantly in females, these lymphomas develop in the gastrointestinal tract (usually stomach, rarely intestine), salivary glands, respiratory tract, thyroid, ocular adnexae (conjunctiva, lacrimal gland, orbit), breast, liver, genitourinary tract, thymus, skin, and dura, some of these sites not being mucosal. There is often a history of a chronic inflammatory disorder with an auto-immune component (Helicobacter pylori gastritis, Sjögren’s syndrome, Hashimoto’s thyroiditis, borreliosis). MALT lymphomas arise in acquired lymphoid tissue and are dependent on a local chronic immune response, at least in their early phase. They are classically indolent localised tumours. Dissemination at diagnosis is not rare (stage IV 32% with 17% of bone marrow involvement), but there is a high response rate to treatment (surgery, radiotherapy, chemotherapy) and a long survival. Regression of gastric low-grade MALT lymphoma after eradication of H. pylori is possible in 70% of the cases when the tumour is localised (stage IE) and superficial, but there is no response in deeply invasive or transformed cases. Whatever the localisation, relapses are frequent, usually late, occurring either in the site of origin or other extranodal sites (perhaps with specific homing) or in lymph nodes. Transformation to an aggressive large B-cell lymphoma may occur. The diagnosis of transformed cases, sometimes difficult from small endoscopic biopsies, is defined by the presence of sheets of large cells (>20 cells); probably cases with more than 5-10% of large cells have a worse prognosis.

without BCL2 rearrangement and bcl2 expression. The differential diagnosis from cutaneous MZL, more frequent, is difficult.

• diffuse lymphoma of follicular type (diffuse centre cell lymphoma), composed of centrocytes, with a minor component of centroblasts, very rare. Immunophenotyping is necessary for the diagnosis, which is difficult.

The postulated normal cell counterparts of FL are follicle centre (germinal centre) B-cells.
Nodal marginal zone B-cell lymphoma, with or without monocytoid B-cells. The characterisation from this subtype among small B-cell lymphomas is still under discussion. The main problem is with tumours showing a plasmacytic differentiation, and there seems to be a nearly complete overlap with immunocytoma. It can be difficult to make a diagnosis in lymph nodes from other small B-cell lymphomas, which sometimes have a marginal-zone pattern or contain monocytoid B-cells (MCL, FL, SLL) or with a nodal spread of MALT lymphoma. And the transformation to large cell lymphoma at diagnosis is more frequent than in other subtypes.

The initial clinical description of monocytoid B-cell lymphoma concerned elderly women, often with Sjögren’s syndrome and a localised tumour (some cases possibly corresponding to a nodal spread of an occult MALT salivary lymphoma). In fact, advanced-stage disease is frequent with peripheral and abdominal lymphadenopathy and the prognosis is poorer than in MALT and splenic MZL.

Splenic MZL, with or without villous lymphocytes. This is probably the only primary low-grade B-cell lymphoma occurring in the spleen. Patients are adults, presenting usually with prominent splenomegaly, without peripheral lymph node involvement, and sometimes with a M component, an autoimmune thrombocytopenia or anaemia. The blood and bone marrow are involved. The circulating tumoral cells are not always villous lymphocytes but can be small atypical non-villous B-cells. The clinical course is indolent.

The diagnosis can be very difficult in the spleen, where MCL and FL display the same micronodular pattern. It is easier in hilar lymph nodes, but border-line cases of SMZL CD5+ with t(11;14) exist.

The splenic histology of SLVL, defined by cytology of blood involvement, is still debated. Probably, it is not always similar to SMZL, as described by Isaacson, but can be different, mimicking hairy cell leukaemia, as described by Neiman. The question is still open: is SLVL a variant of splenic MZL or not?

In conclusion, characterisation of small B-cell lymphomas is still underway and will probably result in a more precise diagnosis of the different variants and subtypes, in a few years. Identification of prognostic criteria with biological and clinical relevance among single entities is of great importance. These criteria will include not only clinical, morphological and immunologic features but also genetic and molecular data, reflecting the mechanisms of lymphoma triggering and tumour progression.

References
Current status and perspectives of therapy for follicular lymphomas

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Epidemiology and clinical course

Follicular lymphomas (FL) are world-wide the second most frequent subtype of nodal lymphoid malignancies. The incidence of follicular lymphomas has rapidly increased during the last few decades, rising from 5-6 new cases per 100,000 inhabitants per year during the 50s to more than 13-15 cases per 100,000 inhabitants per year recently. Increasing evidence suggests that this development is at least partially caused by environmental factors among which pesticides, dyes, cotton wool particles and others are being discussed.

The clinical course of follicular lymphomas is characterised by a slowly progressive enlargement of lymph nodes, an impairment of haematopoesis, an increased susceptibility to infections and the appearance of B symptoms. In 40% of cases follicular lymphomas transform to a high grade lymphoma, mostly of centroblastic or immunoblastic subtype.

Pathogenesis and Biology

Follicular lymphoma was the first malignancy in which it was demonstrated, that a malignant transformation could be caused not only by disturbances of proliferation and differentiation but rather by an impairment of apoptosis. The molecular basis for this event is a translocation between chromosomes 14 and 18 leading to the fusion of the bcl-2-gene on chromosome 18 to the gene for the immunoglobulin heavy chain on chromosome 14. This fusion leads to the overexpression of bcl-2 and an impairment of apoptosis. The thereby prolonged survival of lymphoma cells makes them susceptible to subsequent molecular events, which ultimately lead to the development of malignant lymphomas. bcl-2 overexpression is also associated with an increased resistance to cytostatic agents and corticosteroids, which may explain the limited anti-lymphoma activity of most available anti-neoplastic drugs.

Therapy

Initial chemotherapy

The treatment of follicular lymphomas is guided by the extent of the disease. In the early stages I and II the treatment of choice comprises extended field or total nodal irradiation with a total dose of 30 Gy. This approach can be applied in the hope of achieving a cure. A survey of published results with observation times of more than 5 years indicates a disease-free survival of 60-70% at 5 years and of 40-50% at 10 years (Table 1). In spite of these promising results it must be emphasised, that less than 20% of follicular lymphomas are diagnosed at such early stages. The initiation of potentially curative radiotherapy thus demands careful assessment of the disease stage in order to exclude a more advanced stage of the disorder.

The majority of follicular lymphomas are diagnosed in advanced stages III and IV. No curative treatment has yet been established for this situation. Conventional therapeutic approaches are carried out with palliative intent and should only be initiated upon signs of progression, the existence of B symptoms, an impairment of the haematopoietic system or the existence of bulky disease.

Conventional treatment modalities consist of cytoreductive combination regimens of moderate intensity such as COP, PmM or CHOP. The use of nucleoside analogues, particularly fludarabine, during initial therapy is currently being assessed in prospective randomised trials and cannot yet be recommended for first line treatment.

Complete and partial remissions can be achieved in 60-80% of cases using standard chemotherapy. The rate of complete remissions can be enhanced to up to 50-70% using more intensive treatment. However, this beneficial effect does not translate into longer lasting remission or improved overall survival this last having remained unchanged over the last few decades (Table 2). Hence, the primary reduction of the lymphoma cell mass is not the major challenge of therapy which should rather be maintenance of remission and long term suppression of lymphoma regrowth.

Interferon-α

Attempts to prolong remission duration and to increase the efficacy of initial treatment have particularly focused on the addition of interferon-α either simultaneously to initial cytoreductive chemotherapy or consecutively following successful initial treatment for maintenance therapy. Both approaches have been investigated in prospective randomised trials. As indicated by Table 3 the addition of interferon-α to initial cytoreductive chemotherapy did not result in a beneficial effect when interferon-α was combined with single alkylating agents or combinations of them. In contrast, prolongation of the disease-free survival was obtained when interferon-α was added to anthracycline containing combinations, as demonstrated by the Groupe D’Études des Lymphomes
The effect of interferon-α maintenance treatment after successful initial chemotherapy was assessed in a different series of studies. In all so far published series a tendency for a prolongation of the progression free interval was reported (Table 4). However, the study carried out by GLSG was the only one to show a significant difference. This somewhat unique finding results from the fact that relatively intensive initial cytoreductive chemotherapy was given by the GLSG, but probably more importantly from the fact that interferon-α maintenance treatment was given without a time limitation until the disease progressed or intolerable toxicity occurred.19,20

The beneficial effect of interferon-α maintenance treatment also emerged from a meta-analysis in Table 3. Follicular lymphomas. Combination of interferon-α with chemotherapy.

Table 1. Indolent lymphomas. Radiotherapy for stage I-III.

<table>
<thead>
<tr>
<th>Authors</th>
<th># Observation time</th>
<th>Relapse-free survival (%)</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fuller et al., 1975</td>
<td>62</td>
<td>5</td>
<td>35</td>
</tr>
<tr>
<td>Gospodarowicz et al., 1984</td>
<td>190</td>
<td>10</td>
<td>53</td>
</tr>
<tr>
<td>Panyani et al., 1983</td>
<td>124</td>
<td>10</td>
<td>54</td>
</tr>
<tr>
<td>Sutcliffe et al., 1985</td>
<td>220</td>
<td>10</td>
<td>45</td>
</tr>
<tr>
<td>Mc Laughlin et al., 1981</td>
<td>42</td>
<td>5</td>
<td>40</td>
</tr>
<tr>
<td>Richards et al., 1989</td>
<td>57</td>
<td>5</td>
<td>79</td>
</tr>
<tr>
<td>Epelbaum et al., 1992</td>
<td>35</td>
<td>10</td>
<td>57</td>
</tr>
<tr>
<td>Sack et al., 1992</td>
<td>145</td>
<td>3</td>
<td>76</td>
</tr>
<tr>
<td>Jacobs et al., 1993</td>
<td>34</td>
<td>15</td>
<td>40</td>
</tr>
<tr>
<td>Yahalom et al., 1993</td>
<td>26</td>
<td>7</td>
<td>66</td>
</tr>
<tr>
<td>Mc Ma et al., 1996</td>
<td>177</td>
<td>20</td>
<td>37</td>
</tr>
<tr>
<td>Cox et al., 1984</td>
<td>29</td>
<td>5</td>
<td>61</td>
</tr>
<tr>
<td>Panyani et al., 1984</td>
<td>66</td>
<td>10</td>
<td>40</td>
</tr>
<tr>
<td>Sack et al., 1992</td>
<td>26</td>
<td>3</td>
<td>69</td>
</tr>
</tbody>
</table>

Table 2. Cytoreductive chemotherapy for stages III and IV indolent lymphomas.

<table>
<thead>
<tr>
<th>Therapy</th>
<th># RR (%)</th>
<th>5-yrs DFS (%)</th>
<th>5-yrs OS (%)</th>
<th>Authors</th>
</tr>
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<tr>
<td>Cb</td>
<td>33</td>
<td>33</td>
<td>0</td>
<td>60</td>
</tr>
<tr>
<td>CP</td>
<td>48</td>
<td>64</td>
<td>22</td>
<td>62</td>
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<tr>
<td>CP</td>
<td>152</td>
<td>36</td>
<td>n.a.</td>
<td>49</td>
</tr>
<tr>
<td>Mitox</td>
<td>21</td>
<td>100</td>
<td>50</td>
<td>85</td>
</tr>
<tr>
<td>CDP</td>
<td>35</td>
<td>91</td>
<td>n.a.</td>
<td>49</td>
</tr>
<tr>
<td>COP</td>
<td>84</td>
<td>57</td>
<td>18</td>
<td>60</td>
</tr>
<tr>
<td>COP</td>
<td>99</td>
<td>85</td>
<td>n.a.</td>
<td>49</td>
</tr>
<tr>
<td>COP</td>
<td>248</td>
<td>80</td>
<td>n.a.</td>
<td>49</td>
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<tr>
<td>CnM</td>
<td>93</td>
<td>86</td>
<td>n.a.</td>
<td>49</td>
</tr>
<tr>
<td>COP vs CnM</td>
<td>246</td>
<td>83 vs 84</td>
<td>n.a.</td>
<td>49</td>
</tr>
<tr>
<td>COP-Bleo</td>
<td>77</td>
<td>71</td>
<td>29</td>
<td>50</td>
</tr>
<tr>
<td>CHOP</td>
<td>415</td>
<td>64</td>
<td>n.a.</td>
<td>35</td>
</tr>
<tr>
<td>CHOP</td>
<td>127</td>
<td>60</td>
<td>n.a.</td>
<td>49</td>
</tr>
<tr>
<td>CHOP</td>
<td>23</td>
<td>91.3</td>
<td>n.a.</td>
<td>49</td>
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<tr>
<td>CHOP-Bleo</td>
<td>75</td>
<td>72</td>
<td>38</td>
<td>57</td>
</tr>
<tr>
<td>CHOP-Bleo</td>
<td>22</td>
<td>81</td>
<td>62</td>
<td>81</td>
</tr>
<tr>
<td>CHOP-Bleo</td>
<td>96</td>
<td>77</td>
<td>28</td>
<td>65</td>
</tr>
<tr>
<td>CHOP-Bleo + CMED</td>
<td>108</td>
<td>72</td>
<td>60</td>
<td>62</td>
</tr>
<tr>
<td>M-BACOD</td>
<td>18</td>
<td>56</td>
<td>22</td>
<td>46</td>
</tr>
<tr>
<td>m-BACOD</td>
<td>86</td>
<td>69</td>
<td>31</td>
<td>62</td>
</tr>
<tr>
<td>MACOP-B</td>
<td>125</td>
<td>84</td>
<td>25</td>
<td>70</td>
</tr>
<tr>
<td>CAP-BOP</td>
<td>59</td>
<td>49</td>
<td>16</td>
<td>40</td>
</tr>
<tr>
<td>ProMACE-CytaBOM</td>
<td>20</td>
<td>95</td>
<td>n.a.</td>
<td>16</td>
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<tr>
<td>Fludara</td>
<td>194</td>
<td>69</td>
<td>n.a.</td>
<td>16</td>
</tr>
<tr>
<td>Fludara</td>
<td>54</td>
<td>65</td>
<td>n.a.</td>
<td>16</td>
</tr>
<tr>
<td>Fludara</td>
<td>88</td>
<td>83</td>
<td>n.a.</td>
<td>16</td>
</tr>
<tr>
<td>Fludara+HD</td>
<td>85</td>
<td>76</td>
<td>n.a.</td>
<td>16</td>
</tr>
<tr>
<td>Fludara</td>
<td>71</td>
<td>62</td>
<td>n.a.</td>
<td>16</td>
</tr>
<tr>
<td>Cladribine</td>
<td>55</td>
<td>88</td>
<td>n.a.</td>
<td>16</td>
</tr>
<tr>
<td>Cladribine</td>
<td>44</td>
<td>64</td>
<td>n.a.</td>
<td>16</td>
</tr>
</tbody>
</table>

RR = remission rate.

De l’Adulte (GELA).18

The effect of interferon-α maintenance treatment after successful initial chemotherapy was assessed in a different series of studies. In all so far published series a tendency for a prolongation of the progression free interval was reported (Table 4). However, the study carried out by GLSG was the only one to show a significant difference. This somehow unique finding results from the fact that relatively intensive initial cytoreductive chemotherapy was given by the GLSG, but probably more importantly from the fact that interferon-α maintenance treatment was given without a time limitation until the disease progressed or intolerable toxicity occurred.19,20

The beneficial effect of interferon-α maintenance treatment also emerged from a meta-analysis in

5th Congress of the European Haematology Association - Educational Book
Myeloablative treatment and stem cell transplantation

Despite these achievements no currently available treatment modality has been demonstrated to have a beneficial effect on overall survival. In particular, no treatment has yet been shown to have curative potential. Assuming that radiotherapy might have the potential to eradicate follicular lymphoma the strategy of myeloablative radiochemotherapy followed by stem cell transplantation has recently gained increasing interest. A series of phase II studies have emphasised the hope of prolonging disease control and possibly even a cure but longer follow-up of these trials also indicated a potential increase in secondary neoplasms such as myelodysplastic syndromes and secondary acute myeloid leukaemias.\(^\text{22-25}\)

The GLSG recently completed the first world-wide, randomised, prospective trial of myeloablative radiochemotherapy followed by stem cell transplantation has recently gained increasing interest. A series of phase II studies have emphasised the hope of prolonging disease control and possibly even a cure but longer follow-up of these trials also indicated a potential increase in secondary neoplasms such as myelodysplastic syndromes and secondary acute myeloid leukaemias.\(^\text{22-25}\)

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Immunotherapy

Increasing insights into the physiology of the immune system, and the pathogenesis of malignant lymphomas and improved technologies have recently opened up new perspectives for immunotherapeutic strategies. These include the application of anti-lymphoma antibodies from among which anti-CD20 monoclonal antibodies directed against lymphoma antigenes can also be used for targeted treatment when coupled with radioisotopes such as \(^{131}\)iodine or yttrium. In a series of extended phase II studies highly encouraging results were reported in patients with relapsed or refractory follicular lymphomas being treated either with myeloablative doses followed by stem cell transplantation or after non-myeloablative radioisotope intensity.\(^\text{34,36}\)

New and promising strategies are trying to take advantage of the specific molecular aberration and bcl-2-overexpression by using therapeutic antisense oligonucleotides. Results of the first clinical trials are promising but it is too early to judge the clinical value of such approaches.\(^\text{39}\)

In summary, recent insights into the biology of malignant lymphomas and follicular lymphomas in particular have opened up the way to new therapeutic strategies with increased specificity and enhanced activity. Hence, the hope appears to be justified that more effective and potentially even curative strategies may become available within the near future for patients suffering from follicular lymphomas.

### References


### Table 4. Follicular lymphomas. IFN-α maintenance.

<table>
<thead>
<tr>
<th>IFN-α</th>
<th>Dose and duration</th>
<th>IFN-α %</th>
<th>Control %</th>
<th>Time-point</th>
<th>Authors</th>
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<tbody>
<tr>
<td>2 mil.</td>
<td>3x/week 2 yrs.</td>
<td>53</td>
<td>41</td>
<td>4 yrs, p = 0.08</td>
<td>Peterson et al. 1997*</td>
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<tr>
<td>3 mil.</td>
<td>3x/week 1 yr.</td>
<td>43</td>
<td>35</td>
<td>3 yrs, n.s.</td>
<td>Hagenbeek et al. 1996</td>
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<tr>
<td>5 mil.</td>
<td>3x/week cont.</td>
<td>46</td>
<td>22</td>
<td>4 yrs, p &lt; 0.005</td>
<td>Hiddemann et al. 1996</td>
</tr>
<tr>
<td>5 mil.</td>
<td>3x/week 1 yr.</td>
<td>62</td>
<td>25</td>
<td>8 yrs, p &lt; 0.01</td>
<td>Aules et al. 1996*</td>
</tr>
<tr>
<td>3 mil.</td>
<td>3x/week 1 yr.</td>
<td>66</td>
<td>28</td>
<td>4 yrs, p = 0.05</td>
<td>Rohatiner et al. 1997*</td>
</tr>
</tbody>
</table>

*Only CR patients.
32. Grillo-Lopez AJ, White CA, Shen D et al. Pilot efficacy studies of rituximab in combination with...


Treatment of non-follicular indolent lymphomas

BERTRAND COIFFIER
Hospices Civils de Lyon and Université Claude Bernard, Lyon, France

The recently described World Health Organization classification of haematopoietic neoplasms is based on morphological, immunological, and genetic characterization of the different lymphomas. The improvement in lymphoma definition was particularly important for diffuse small B-cell lymphoma entities. These lymphomas were classified under the name of type A in the Working Formulation with two subtypes, small lymphocytic consistent with chronic lymphocytic leukaemia (CLL) and small lymphocytic, plasmacytoid, but some were found in the diffuse small cleaved cell, diffuse mixed, and even follicular small cell categories. In the Kiel classification, these lymphomas were classified under the names of B CLL, B prolymphocytic leukaemia, lymphoplasmacytoid immunocytoma, and lymphoplasmacytoid immunocytoma. Under these different names, pathologists included different entities that have in common a diffuse infiltration with small cells having the appearance of small lymphocytes, prolymphocytes, paraimmunoblasts, plasmacytoid lymphocytes, plasma cells, and all their possible intermediate cells. Years ago, entities such as mantle cell lymphoma or marginal zone lymphoma were often included under these names but these are now recognised as different lymphomas.

Although pathologists may understand what is designated under these names, the definition of each entity remains incomplete and their relationship with CLL, Waldenström’s macroglobulinaemia (WM) and other indolent lymphom proliferative diseases remains uncertain. The clinical aspect of these lymphomas is not well known, their true prognosis is not determined, and recommended therapeutic options are undefined. We present here a review of the current position on this not completely settled subject.

The different entities

Non-follicular lymphomas are defined as indolent because patients have a long history of small lymph nodes that grow then spontaneously regress or of high lymphocyte counts before diagnosis. This characteristic is shared with follicular lymphomas but this latter entity is well defined by the characteristic pattern of the infiltration, cell appearance, positivity for CD10 antigen, and t(14:18) or bcl-2 rearrangement. In this review, only non-follicular B-cell lymphomas i.e. those presented in Table 1 will be discussed and the associated review. Although some T-cell lymphomas may be considered as indolent, their clinical presentation and characteristics are completely different from the B-cell lymphomas and they must be considered as different entities. Because of these differences, the indolent T-cell lymphomas will not be reviewed here. Figure 1 presents the incidence of these indolent lymphomas in our department in comparison with other lymphomas.

All these lymphomas have a propensity to increased histological aggressiveness with recurrences. This is translated as either a transformation into a proliferation of large cells or non-cleaved non-Burkitt’s cells, as in small lymphocytic lymphoma (SLL) or lymphoplasmacytic lymphoma (LPL), or by an increase of mitosis and/or of number of cells in S phase, as in mantle cell lymphoma (MCL). This transformation is often associated with a large tumour mass, a decrease in performance status (PS), a high LDH level, additional genetic abnormalities such as c-myc activation, loss of p16 expression, or p53 inactivation, refractoriness to treatment, and a poor outcome. In some patients the diagnosis is only made at the time of this accelerated course or at the time of transformation. In these cases, if the biopsy was taken only from the largest tumour, the lymphoma may be misdiagnosed as a large cell lymphoma and the correct diagnosis may only be made from a review by an expert lymphoma pathologist or from biopsy of other lymph nodes or bone marrow which show the small cell part of the tumour.

The clinical characteristics

No specific clinical presentation is associated with the majority of these lymphomas. Patients may present with four initial pictures: a localised extranodal tumour such as in mucosa-associated lymphoid tissue (MALT) lymphomas, a disseminated enlargement of lymph nodes, an enlarged spleen, or a leukaemic disease. The main characteristics are the indolent nature of the lymphoma with a long history of waxing and waning lymph nodes or of increased lymphocyte counts, the absence of B symptoms, and a normal performance status. This favourable aspect does not translate into a good outcome in all cases.

Small lymphocytic lymphoma and lymphoplasmacytic lymphoma

These lymphomas are the nodal counterpart of chronic lymphocytic leukaemia (CLL). They are not very frequent and seem to represent 6% to 8% of all lymphomas in the different series although their true incidence is not known because their limits are not well defined. Patients usually have peripheral lymphocytic lymphomas.
phadenopathy with splenomegaly, without there being much difference between SLL and LPL patients. Localised cases with only one or two enlarged lymph nodes or extranodal disease are very rare. Although the leukaemic picture is not predominant, a large proportion of these patients have bone marrow involvement and more than 50% of them have blood involvement. However, if lymphoma cells are present in the blood, lymphocyte count is usually less than 10×10^9/L. Involvement of extranodal sites is rare, at least at diagnosis, but slightly more frequent in cases of LPL. An M component is more frequent in LPL cases but it is rarely predominant as in marginal zone lymphoma (MZL). Table 2 shows the clinical characteristics of these patients included in the non-Hodgkin’s Lymphoma Classification Project. In our experience, the morphological aspect of this lymphoma does not allow clear-cut separation from CLL, so we consider a diagnosis of lymphoma when there is a discrepancy between the tumour volume in lymph nodes and the leukocyte level in blood or a nodal or extranodal involvement without high lymphocyte counts in blood. In fact this distinction does not seem important because most of the patients diagnosed disease as having lymphoma have recurrences that look like CLL. Moreover, it does not currently affect treatment choices.

Marginal zone lymphomas

This entity represents a recent improvement of the lymphoma classification, being first described as a provisional entity in the REAL classification then definitively settled in the WHO classification. Although MALT lymphomas are now recognised, other subtypes have been classified under different entities in the past and this may still occur if a diagnosis is not reviewed by a haematopathologist. The WHO classification recognises three clinical presentations: extranodal MZL or MALT lymphomas, nodal MZL, and splenic MZL, but cases with more disseminated disease or just bone marrow involvement may be observed. This lymphoma is the third most frequent lymphoma (Figure 1) and its frequency will probably increase when large cell lymphomas with some small cell component (composite lymphoma in the WHO classification) are classified according to the small cell component. Most patients with MALT lymphoma have histories of chronic antigenic stimulation or auto-immune disease. Most of them present with localised stage...
extranodal disease. Although the stomach is the most common site, various other locations have also been described such as lung, skin, thyroid, orbit, salivary glands and other parts of the gastrointestinal tract. Figure 2 presents an update of our experience of the frequency of the different MALT lymphomas. The gastrointestinal tract was involved in 50% of cases, with the stomach being involved in 39% of all cases. Among the non-gastrointestinal sites, skin, lung, salivary glands, and orbit were each affected in 10% of the cases. There are very few differences in the clinical characteristics at presentation between gastrointestinal and non-gastrointestinal cases. Dissemination of the tumour to other MALT sites, spleen or bone marrow is present at diagnosis in 33% of the cases, but is more frequent in recurrent disease. Contrariwise, patients with bone marrow, spleen, and lymph node involvement have an end-stage lymphoma. An M component is present in 20% to 40% of MZL patients; these cases were often diagnosed as having Waldenström's macroglobulinaemia in the past, although all cases of WM were not MZL.

Mantle cell lymphoma

This lymphoma represents 6% to 8% of all lymphomas. It occurs more frequently in older adults, and more commonly in men. It is usually widely spread at diagnosis, with involvement of lymph nodes, spleen, bone marrow, peripheral blood, and extranodal sites, especially the gastrointestinal tract (lymphomatous polyposis). It is initially a moderately aggressive disease but becomes progressively more aggressive with inefficacy of treatments.

Prognostic indicators for survival and time to progression

Outside the lymphoma entity, various clinical and biological features have been identified as being associated with response to treatment and survival. However, most studies have concentrated on large cell lymphomas or follicular lymphoma and consequently very few prognostic indicators have been validated in indolent non-follicular lymphomas. Nevertheless, in our experience, most of the prognostic parameters associated with the ability to achieve a complete response (CR) and with a long survival that have been described for other lymphoma entities may be applied to the indolent non-follicular lymphomas.

Parameters associated with high tumor burden

A large tumour mass has long been recognised as an important adverse parameter even though the methods of assessment have been quite different from one study to another. Parameters associated with large tumour mass and a poorer outcome are: large number of nodal sites, large number of extranodal sites, certain specific locations, large tumour diameter, disseminated stage, high serum LDH level and high β2-microglobulin level. Ann Arbor stage is not very satisfactory for these lymphomas because nearly all of them, except MALT lymphomas, are disseminated with bone marrow involvement (Table 2). Even for MALT lymphoma patients, those with a truly localised disease seem to have the same outcome as patients with disseminated disease at diagnosis. The presence of more than one extranodal site has been associated with poor outcome in aggressive lymphoma and follicular lymphoma patients but this has never been analysed in those with indolent lymphomas. As most such patients have bone marrow and blood involvement, this parameter may lose its prognostic value. Moreover, in MZL, blood involvement is a characteristic of splenic lymphomas and it is associated with a better outcome as shown in Figure 3. Conversely, blood involvement is frequent in MCL too, but there it is associated with a poorer outcome. Above normal levels of LDH have been identi-
fied as a prognostic factor in lymphoma patients in almost all published prognostic analyses and this is true for these indolent lymphomas. The putative importance of the β₂-microglobulin level has been recognised and applied to prognostic analyses in several centres, and, like LDH level, it appears to be one of the parameters that predict the risk of progression.

Parameters associated with patient’s response to the tumour
These parameters include presence of B symptoms, poor performance status, low serum albumin and low haemoglobin levels, which are all well correlated. They probably reflect the same phenomenon, cytokine secretion by either tumour cells or the patient’s immune cells in response to the tumour. Their presence is associated with poor outcome in all these indolent lymphomas.

Parameters associated with the patient’s ability to tolerate the treatment
Outcome for lymphoma patients may differ depending on whether they already have diseases not related to the lymphoma, and on their age at the time of diagnosis. The cut-off between young and old patients is between 60 and 65 years for patients with DLCL. However, although tolerance to treatment decreases with age, it is not perfectly correlated to chronological age but rather with physiological age. In an international review, the oldest patients were predominantly female and had a poorer performance status but other main prognostic parameters such as stage, number of extranodal sites, or LDH level were not statistically different according to age distribution.8,19

Prognostic indices
Because of the numerous prognostic parameters described, an international classification system based on clinically relevant prognostic factors was developed for DLCL.18 This International Prognostic Index (IPI) is based on age, tumour stage, serum LDH level, performance status, and number of extranodal disease sites, and makes it possible to identify four risk groups according to the number of adverse parameters. In young patients an age-adjusted IPI based on tumour stage, serum LDH level and performance status also identified four risk groups. In both models, the increased risk of death was the result of both a lower rate of complete responses and a higher rate of relapses. The IPI may also be effectively applied to patients presenting with other lymphoma entities.14,20 However, the IPI was not been validated for MZL, SLL or LPL patients. Moreover, while it defined groups of patients with MCL with statistically significant differences in survival, this difference may not be clinically relevant as shown in Figure 4, the difference between poor risk and good risk patients being small.

Treatment and outcome
A literature review concerning the treatment of these lymphomas is of very limited use because these entities have only been well defined recently. MZL was not recognised in the Working Formulation or the Kiel Classification so cases with diagnosed as such nowadays were previously classified as small lymphocytic, diffuse small cleaved cell, diffuse mixed, or even diffuse large cell lymphomas in the Working Formulation and in the CLL subtype or immunocytoma in the Kiel Classification. It is clear that retrospective analyses cannot describe the clinical presentation and outcome of these patients, let alone indications on how to treat them. Here, we will review the most important studies on the treatment of these entities, making some assumptions on what the different authors described in old studies, and we will present some results from patients treated in our department. None of the recommendations proposed here can be considered as definitive and most of them will require prospective randomised studies to confirm their value.

Treatment alternatives for individual patients and the design of new therapeutic regimens must take into account histological subtype and the presence of adverse prognostic factors. As an example, Figure 5 shows the survival of patients with indolent lymphoma treated in the author’s centre according to histological subtype. We consider two standard treatments for these patients: intermittent chlorambucil (16 mg/m²/day for 5 days every month) and the CHOP regimen (cyclophosphamide 750 mg/m², doxorubicin 50 mg/m², vincristine 1.4 mg/m², and prednisone 40 mg/m² every 3 weeks for 8 cycles) used for more than 20 years.21,22 When possible, we try to compare the merits of more recent therapies to those of these standard treatments.

Small lymphocytic lymphoma
For many years the treatment for most SLL/LPL patients was single agent chemotherapy (chlorambucil or cyclophosphamide) or a multidrug regimen with or without doxorubicin (CVP: cyclophosphamide + vincristine + prednisone, or CHOP). However, few prospective trials have been designed specifically for these patients and the best therapeutic approach is not yet known. Very few patients reached complete remission, but most of them responded to treatment,
and had a median time to failure (TTF) of 3-5 years. Ultimately, the disease progressed in all patients, often with a leukaemic picture, sometimes with histologic transformation, and all died after a median overall survival of 5-8 years.23-25 However, most of these retrospective analyses were designed to define the differences between SLL and LPL and not between these lymphomas and the other indolent entities. Their conclusions concerning the best treatment, if any existed, were not validated. All these analyses described a more aggressive pattern for LPL than for SLL or CLL but MZL patients were not recognized and some patients with MZL were included under the name of LPL.

Fludarabine has recently proved its efficacy in phase II trials:26 35 LPL patients were specifically identified and their slides were all reviewed. The response rate was 63% with 14% CR; median time to progression was 2.5 years. Small numbers of SLL/LPL patients were included in phase II trials of cladribine and similar results have been reached,27 although hematological toxicity may be more substrated and the occurrence of severe infection more common. Ongoing trials are testing the combination of these agents with mitoxantrone, cyclophosphamide, and/or cytarabine.28,29 In the first studies, the effect of rituximab was low with less than 15% of patients responding to this treatment30 but its role need to be re-considered in well-defined studies.

Initially considered as low-grade lymphomas in the Kiel classification or the Working Formulation, these patients have a shorter survival than most of those with the other B-cell lymphomas: median failure-free survival was around 2 years and median survival less than 5 years in the Non-Hodgkin’s Lymphoma Classification Project.19

Our current strategy is to propose radiotherapy for localised patients with disease; 12-18 months of chlorambucil (16 mg/m²/day x 5 days a month) for patients with disseminated disease who have no adverse prognostic factors; fludarabine (25 mg/m²/day x 5 days a month) for six to nine courses for more patients with advanced disease; 8 courses of CHOP for patients with a high percentage of large cells, poor IPI, or a suspicion of transformation. More intensive therapy with total-body irradiation and autologous stem cell transplantation (ASCT) may be proposed for young patients with histological transformation and for relapsing patients but the role of this strategy is not yet defined. We urgently need prospective trials to identify criteria associated with poor outcome and to specify the role of the above mentioned therapies.

Mantle cell lymphoma
MCL patients are resistant to current therapeutic options.14,31 They often present partial regression of the lymphoma for 6-18 months which then progresses and the patients die after a median overall survival of 3-4 years. While in one randomised trial, CHOP as first line treatment was shown not to be associated with a longer survival than CVP, CHOP or high-dose cytarabine is generally thought to be associated with a better response rate.32,33 The first studies with fludarabine did not give better results.26 However, rituximab has been associated with good results in phase II trials:34 the response rate to rituximab therapy was 37% in relapsing patients and in de novo disease, which is higher than with any other drug alone. In combination with CHOP, response may be as high as 96%.35 However, even patients in complete remission relapsed with a median TTF not really different from with CHOP alone. The definitive role of these agents in the treatment of MCL patients has not yet been determined by randomised trials. High-dose therapy with ASCT has been associated with long disease-free survival in one trial but this was not confirmed by others. The combination of in vivo purge with rituximab plus high-dose therapy may be associated with a longer survival but this, too, needs to be confirmed.15

Outside randomised prospective trials, the following strategy is recommended: patients with localised disease may be treated with involved field radiotherapy; elderly patients without adverse prognostic parameters may be treated with chlorambucil or CVP initially or after disease progression; those with adverse prognostic parameters should be treated with CHOP; young patients may be treated with CHOP or DHAP and, in cases of good response, high-dose therapy with total body irradiation. The place of rituximab needs to be tested in this setting, particularly as in vivo purge before harvesting peripheral stem cells.
Marginal zone lymphomas

Patients with localised MALT lymphoma are usually treated with surgery or local radiotherapy, depending on the disease site, but no prospective trial has validated these options. Chlorambucil or multidrug regimens yielded the same results in patients with more advanced disease. No therapeutic strategy has been recommended for the 35% of patients with disseminated disease at diagnosis or with recurrent disease. Although it was recently demonstrated that localised gastric MALT lymphoma may regress after antibiotic treatment of Helicobacter pylori infection, the effect of this treatment on disseminated gastric MALT lymphoma or non-gastric MALT lymphoma is not yet known. Until the conclusion of prospective trials, the treatment of these patients may be summarised as follows. Patients with localised non-gastric MALT may be treated with surgery, local radiotherapy, or chlorambucil. Currently, we prefer using chlorambucil (16 mg/m² per day, 5 days every month) for 6-12 months because of the long-term problems observed with radiotherapy, and because of surgery sequelae. Patients with disseminated MALT lymphoma should be treated with chemotherapy; fludarabine and chlorambucil are equally effective in obtaining a complete response. However, in patients with a large tumour mass or a substantial large cell component, CHOP chemotherapy must be used. The place of rituximab in the treatment of these patients is not known but a large prospective trial is warranted.

Patients with MZL, splenic type, with or without villous lymphocytes, are often aged and have predominantly splenic disease. They may be treated with splenectomy in case of large splenomegaly or initially not be treated at all. In our experience, splenectomy alone or an initial “watch-and-wait” policy in a selected group of patients without adverse prognostic parameters was associated with a long event-free survival. Fludarabine has been associated with a good outcome in a limited number of patients. Younger patients with splenic MZL patients with a high component of large cells, or those with adverse prognostic parameters probably should be treated with a multidrug regimen but, here too, prospective trials are needed to define the best options. In all these patients, a CR is rarely reached and bone marrow and blood involvement often persist but this is not associated with a rapid progression and need not be considered an indication for high-dose therapy. However, when the disease progresses, with enlarged tumour masses, this option may be considered, particularly in young patients.

The treatment options for patients with nodal MZL are not yet defined, mostly because this entity has only recently been defined and very few groups have much experience in treating these patients. Patients with localised disease may be treated with CHOP followed by radiation therapy as for large cell lymphomas. In our experience, patients with disseminated disease have a short TTP but may experience a long survival. Thus, we recommend a multidrug regimen such as the CHOP regimen for patients with disseminated disease. HDT with AST may be considered for patients with a high contingent of large cells, a poor IPI, or in relapse.

Waldenström’s macroglobulinaemia

This entity was described years ago as a lymphoproliferative disease presenting with a high level of IgM monoclonal component, bone marrow infiltration by lymphoplasmacytic cells, splenomegaly, and, in some cases, blood or lymph node involvement. A hyperviscosity syndrome was often observed in patients with the highest IgM levels. This disease was not categorised within the WHO classification but it corresponds to lymphomas with an M component that may be classified as lymphoplasmacytoid, lymphoplasmacytic, or marginal zone lymphomas. In the past, the challenge was to separate patients with disease needing to be treated from those who did not need early treatment or who may be classified as having a monoclonal gammapathy of unknown significance. Now, the challenge is to unravel the different entities that were previously grouped as called Waldenström’s macroglobulinaemia.

The classic therapy of these patients was chlorambucil but although they responded with a decreased M component and decreased nodal or splenic enlargement, no CR was ever reached and all these patients progressed with an event-free survival and overall survival not different from those described for patients with SLL/LPL. In recent years, fludarabine seems to be being associated with a better outcome, but this needs to be correlated with the different entities.

Conclusions

Indolent lymphomas must not be considered as a whole but specific entities must be used to classify the patients before treatment. It is possible that all these entities have to be treated in the same way but this was never demonstrated. In our experience, they do not have the same presentation and are associated with different responses to treatments and different outcomes. Prospective studies are urgently needed to define these entities better, particularly the SLL/LPL, and to define what may be the best therapeutic options. Outside clinical trials, minimal staging must include cytological and histological analyses and an immunophenotyping of the cells. In clinical trials, cytogenetic analysis and complete phenotyping must be the standard in order to allow retrospective analyses.

References.

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Chairman: V. Diehl, Cologne

Session 2 – HIGH GRADE NON-HODGKIN’S LYMPHOMA

Therapeutic strategies for aggressive lymphomas

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Definition
The term high-grade non-Hodgkin’s lymphoma (NHL) continues to create confusion since it has been used by pathologists and clinicians alike with different meanings. In the Kiel classification, the term high-grade was used to distinguish those lymphomas dominated by blast-type neoplastic cells from the so-called low-grade NHL, whose dominant neoplastic cell population was of non-blast type, cytotoxic cell morphology. Since there was a considerable, but incomplete overlap between the histological morphology of cytotoxic lymphomas and an indolent clinical course on the one hand and blast morphology of the neoplastic lymphocytes and an aggressive clinical course on the other, the clinical and histomorphological terms were often used indiscriminately. The common usage of the term high-grade lymphoma by different people meaning different things mirrors only one of several ambiguities that kept both basic researchers and pathologists from both sides of the Atlantic from speaking a uniform language. In order to overcome this classificatory babel, haematopathologists from several continents devised a new universal classification scheme. Based on the Kiel classification, the new REAL (Revised European-American Lymphoma) classification groups NHL entities not only according to the morphology of the neoplastic cell population, but also includes immunological and genetic information to subgroup different lymphomas. The WHO is soon to introduce a new classification based on the REAL one.

The usefulness of the REAL classification system was demonstrated by several studies performed by an international panel of expert pathologists and clinicians. The terms indolent, aggressive and very aggressive should be used to distinguish groups of lymphomas with the described clinical courses. In order to avoid further misunderstanding between clinicians and pathologists when using the term high-grade, we propose that the clinical term aggressive lymphomas is used for the entities listed in Table 1.

Therapeutic strategies
Radiotherapy
Aggressive NHL are highly radiosensitive and doses between 36 Gy and 45 Gy appear to be sufficient to eradicate the malignant clone. Although this suggests that radiotherapy alone has curative potential for early stage disease, it must be kept in mind that the BNLI trials have taught us that elderly patients (>70 years of age) with aggressive NHL cannot be cured by radiotherapy alone and that therefore radiotherapy alone should not be used any more to treat early stage aggressive NHL.

The role of combined chemoradiotherapy (combination of a reduced number of chemotherapy cycles with involved-field radiotherapy) for localised stages is still poorly defined. In a trial carried out in a few centres on patients with intermediate and high-grade NHL in stages I and II were randomised to receive either 3 cycles of CHOP chemotherapy with additional aggressive (>45 Gy) involved-field radiotherapy or 8 cycles of CHOP. Disease-free and overall survival were significantly better in patients in the combined modality treatment arm compared to those in the chemotherapy-only arm. Translating these data into clinical practice is difficult since the trial included patients with intermediate-grade lymphomas, a considerable number of cases with exclusively extranodal disease and without measurable disease after biopsy. It is of note that stage II, elevated LDH, age >60 years and decreased performance status turned out to be adverse prognostic factors in that they were associated with significantly worse treatment results. Therefore, the combination of shortened chemotherapy and radiotherapy cannot be recommended for the large majority of patients outside the setting of multicentre trials that are necessary to clarify this important question.

Consolidation radiotherapy given in addition to full-cycle chemotherapy is a frequent practice, but one unfortunately based on limited data. The results of one small randomised trial on 88 patients suggest that radiotherapy given to sites of initial bulky disease can improve relapse-free and overall survival. There is no study that has properly addressed the question of giving radiotherapy to residual masses after chemotherapy (iceberg radiotherapy). Therefore, radiotherapy in combination with chemotherapy for aggressive NHL should preferably be given within the context of an urgently needed prospective clinical trial.

Chemotherapy
The break-through in the treatment of aggressive NHL was achieved more than 20 years ago with the development of modern polychemotherapy, namely the combination of doxorubicin, vincristine, cyclophosphamide and prednisone (CHOP). Encouraged by the demonstration that a considerable proportion...
of patients with aggressive NHL, even in those advanced stages, can be cured by chemotherapy, many modifications of the CHOP regimen were designed and tested in clinical trials. Based on the model calculations of Goldie and Coldman and the dose-intensity concept of Hryniuk and the dose-intensity concept of Hryniuk modified for lymphomas, several phase II trials with so-called second and third-generation chemotherapy regimens were designed. Phase II trials showed CR rates in aggressive NHL rising from 50 to 90% and 5-year survival rates from one to over two-thirds when compared to CHOP. However, the excellent results obtained with these later-generation regimens could not be confirmed in multi-institutional phase-III studies with greater number of patients. A large intergroup trial that included more than 1,200 untreated patients with aggressive (diffuse large cell) NHL which had been initiated by the SWOG compared CHOP with m-BA-COD (methotrexate, bleomycin, doxorubicin, cyclophosphamide, vincristine and dexamethasone), ProMACE-CytarBOM (prednisone, methotrexate, doxorubicin, cyclophosphamide, etoposide, cytarabine, bleomycin, vincristine) and MACOP-B (methotrexate, doxorubicin, cyclophosphamide, vincristine, prednisone, bleomycin). While there was no difference in CR rate, time to treatment failure or overall survival between CHOP and the later-generation regimens, the toxicities of the new regimens were higher, with treatment-related fatalities up to 6% compared to 1% observed in the CHOP arm. Similar results were obtained in a smaller multi-centre trial conducted by the ECOG which found no difference in therapeutic efficacy between m-BA-COD and CHOP.

New treatment strategies: the International Prognostic Factors project

The results of the intergroup trial led to a great disillusionment among lymphoma physicians since CR and cure rates had not changed over a period of nearly 20 years and the belief was taking hold that treatment results were primarily determined by selection of patients rather than by the particular first-, second- or third-generation chemotherapy regimen given.

The identification of factors underlying patient selection in trials carried out in only a few centres became of prime importance, since it was obviously a prerequisite for better understanding of the factors determining the success or failure of chemotherapy. An international group of investigators analysed pretreatment factors of nearly five thousand patients with aggressive lymphoma who had been treated in prospective trials that employed doxorubicin-based chemotherapy regimens. Five pretreatment parameters were found to be of independent prognostic significance: lactate dehydrogenase serum level (normal vs. elevated), age (≤ 60 vs. >60 years), localised stages (I or II) vs. advanced stages (III and IV), number of extranodal sites of involvement (≤ 1 vs. > 1), and performance status (ECOG 0, 1 vs. ≥2). According to the number of risk factors NHL patients present with they can be grouped into four risk groups with significantly different prognosis (Table 2).

The development of the IPI led to the realisation that different strategies might be necessary to improve of results in the different risk groups, and many co-operative study groups have initiated trials pursuing differentiated strategies. Most groups evaluate some form of high-dose chemotherapy for younger patients (<60 or 65 years) and more moderate treatment intensification in the other risk groups and the elderly. Since stage is only one of five risk factors, NHL patients present with there is an ongoing debate as to the prognosis of mediastinal large B-cell lymphomas compared to their non-mediastinal counterparts. There are indications that the prognosis of patients with diffuse large B-cell lymphomas, which account for more than two-thirds of aggressive NHL. There are indications that the prognosis of particular subentities of aggressive lymphomas varies considerably: e.g., it is widely accepted that the T-cell phenotype (with the exception of anaplastic large T-cell lymphomas) is an independent adverse prognostic factor. Similarly, the immunoblastic variant had a worse outcome in a retrospective analysis and there is an ongoing debate as to the prognosis of mediastinal large B-cell lymphomas compared to their non-mediastinal counterparts.

While certain subentities of the subgroup of aggressive lymphomas might be associated with a worse prognosis, there is at present no proven strategy on

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**Table 1. Aggressive lymphomas.**

<table>
<thead>
<tr>
<th>Type of NHL</th>
<th>Subtypes</th>
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<tbody>
<tr>
<td>B-cell NHL</td>
<td>mediastinal large B-cell lymphoma</td>
</tr>
<tr>
<td></td>
<td>intravascular large B-cell lymphoma</td>
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<tr>
<td></td>
<td>primary effusion lymphoma</td>
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<tr>
<td></td>
<td>diffuse large B-cell lymphoma, not otherwise specified</td>
</tr>
<tr>
<td></td>
<td>variants: centroblastic, immunoblastic, T-cell or histiocytic, rich, anaplastic large B-cell</td>
</tr>
<tr>
<td>T-cell NHL</td>
<td>peripheral T-cell lymphoma, not otherwise specified</td>
</tr>
<tr>
<td></td>
<td>natural-killer T-cell lymphoma, nasal and nasal-type</td>
</tr>
<tr>
<td></td>
<td>angioimmunoblastic T-cell lymphoma</td>
</tr>
<tr>
<td></td>
<td>adult T-cell leukemia/lymphoma (human T-cell leukemia virus-1)</td>
</tr>
<tr>
<td></td>
<td>anaplastic large-cell lymphoma (T and null types)</td>
</tr>
<tr>
<td></td>
<td>subcutaneous panniculitis-like T-cell lymphoma</td>
</tr>
<tr>
<td></td>
<td>enteropathy-type intestinal T-cell lymphoma</td>
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<tr>
<td></td>
<td>hepatosplenic T-cell lymphoma</td>
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</tbody>
</table>

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**Table 2. Risk factor groups according to the IPI.**

<table>
<thead>
<tr>
<th>Risk group</th>
<th>No. of risk factors</th>
<th>CR rate</th>
<th>5-year survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>0.1</td>
<td>87%</td>
<td>73%</td>
</tr>
<tr>
<td>Low-intermediate</td>
<td>2</td>
<td>67%</td>
<td>50%</td>
</tr>
<tr>
<td>High-intermediate</td>
<td>3</td>
<td>55%</td>
<td>43%</td>
</tr>
<tr>
<td>High</td>
<td>4.5</td>
<td>44%</td>
<td>26%</td>
</tr>
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how to overcome this disadvantage using specific therapeutic measures. Such information can only be gained by continuing to include as many subgroups as possible in large randomised trials which allow for prospective contrasting analysis of the subgroups and their respective prognosis. It is therefore strongly recommended that rare subgroups are included in large randomised trials covering the full spectrum of aggressive lymphomas.

While considerable experience has been gained with therapeutic approaches for indolent and very aggressive NHL, e.g. predominantly palliative strategies for disseminated indolent lymphomas and ALL-type chemotherapy for most very aggressive lymphomas, there are insufficient data concerning the optimal approach for so-called grey-zone entities and some others which are usually not included in the group of aggressive NHL. These entities include mantle cell lymphomas and follicular lymphomas grade III on one, and Burkitt as well as Burkitt-like NHL on the other side. These lymphomas are often managed with the treatment strategies used for aggressive lymphomas, and attempts to define whether or not they are treated with the appropriate prototype therapy will be extremely difficult. Matched pair analyses of such patients performed by intergroup data pooling are the only way to ensure sufficiently aggressiveness therapy while avoiding overtreatment.

Extranodal non-Hodgkin’s lymphoma

It is generally accepted that lymphomas evolving in extralymphatic organs have distinct natural histories and present with specific clinical features. Even though these differences are not taken into account in the WHO classification nor in the IPI, specific therapeutic approaches have evolved for the treatment, for example, of primary CNS, testicular, gastrointestinal or paraesophageal sinuses lymphomas. An analysis of the specific approaches, e.g. the addition of local radiotherapy and/or the prophylaxis therapy of sanctuaries, such as intrathecal chemotherapy for the prophylaxis of CNS dissemination of testicular lymphomas, is beyond the scope of this discussion. Intergroup trials or matched pair analyses are necessary to generate information about therapeutic procedures justified and necessary for distinct extranodal presentations of aggressive NHL.

Chemotherapy: intensification strategies

The realisation that CHOP is as effective as second- or third-generation regimens and that certain pretreatment characteristics of the patients are more strongly related to outcome than the chemotherapy regimen used has led to differential strategies for improving results of therapy in aggressive lymphomas. Dose intensification and escalation still appear to be the most promising approaches in young patients. The most widely used strategy for young patients in high and high-intermediate risk groups is high-dose chemotherapy. The superiority of high-dose chemotherapy over an intensive conservative chemotherapy regimen (DHAP) in patients with relapsing aggressive NHL in the PARMA trial prompted many investigators to study a similar approach in the primary treatment of chemotherapy-naive patients. In the Parma trial21 patients <60 years of age with aggressive NHL relapsing after an initial complete remission received two cycles of conventional salvage chemotherapy with the DHAP (dexamethasone, cisplatin, cyclophosphamide) regimen. Responders were randomized to receive additional DHAP chemotherapy or high-dose chemotherapy with the BEAC (carmustine, etoposide, cytarabine, cyclophosphamide) regimen, both with involved-field radiotherapy. Patients receiving high-dose chemotherapy had a significantly better event-free survival (46% vs. 12%, p = 0.01) and a slightly better overall survival rate (p = 0.38).

While the Parma trial has established high-dose chemotherapy with stem cell support as the preferred treatment option in young patients with relapsing aggressive NHL, results of this strategy are less convincing in the primary treatment of aggressive NHL. The GELA-LNH-87 trial randomised 3,709 patients with aggressive NHL who had achieved a CR with the ACVD regimen into sequential conventional chemotherapy or high-dose therapy with autologous stem cell support. There were no differences with regard to disease-free and overall survival between patients randomised to conventional consolidation chemotherapy or high-dose chemotherapy.22 However, a retrospective analysis of the trial limited to high/high-intermediate risk patients, showed a benefit for the high-dose chemotherapy group both in terms of relapse-free and overall survival.23 Similar results were observed in an Italian trial.24 Again, while no advantage for the entire group of patients assigned to high-dose chemotherapy was seen, a subset analysis of patients with high-intermediate and high-risk prognostic indexes significantly favoured the high-dose chemotherapy in terms of disease-free survival, yet not overall survival. Similar results were obtained in a randomised trial carried out by the German NHL study group.25 The results of a randomised Dutch trial, in which high-dose chemotherapy given to patients in partial remission after 3 courses of chemotherapy failed to improve freedom from treatment failure and survival over full-course CHOP chemotherapy,26 support these observations. The GELA-LNH93 study of patients with aggressive NHL in high-intermediate and high-risk groups had to be stopped prematurely because conventional chemotherapy was clearly of more benefit than high-dose therapy.

The small randomised trial reported by Gianni et al.27 to date the only one to suggest a benefit of early high-dose chemotherapy in the primary treatment of aggressive NHL. Despite a comparatively high mortality rate encountered during the early phase of the trial in the autotransplant arm, the 38 patients randomised to sequential high dose chemotherapy had a significantly better progression-free survival than the 37 patients in the MACOP-B arm (p = 0.001). The Swiss cooperative group SAKK has initiated a randomised trial to confirm this strategy with a sufficiently large number of patients.It must be kept in mind that Gianni approach is quite different from other high-dose chemotherapy concepts that were pursued in the other trials, since it contains many different modules (e.g. high-dose methotrexate, fractionated high doses). It cannot be excluded that the
benefit of “sequential high dose chemotherapy” is not due to the high drug doses but to these other elements.

The intergroup trial by Fisher et al.12 and the unconvincing results of high-dose chemotherapy in the treatment of chemotherapy-naive patients with aggressive NHL prompted many investigators to pursue dose intensification within the framework of CHOP or CHOP-like regimens. The availability of recombination stimulators of haematopoiesis has made this approach possible without the addition of stem cell support. Phase II trials have shown that considerable escalation is indeed possible both with the CHOP regimen28 and with later-generation modifications of CHOP. The dose of cyclophosphamide could be more than quadrupled (4,000 mg/m² instead of 750 mg/m²), together with a considerable increase of the doxorubicin dose (70 mg/m² instead of 50 mg/m²). While the results of these dose-escalation trials are hard to interpret because of the inclusion of patients with a heterogeneous risk profile, the occurrence of treatment-related MDS and acute leukaemias in this trial (Shipp MA, personal communication) highlights the inherent risk of this strategy of treatment intensification. Moreover, the acute treatment-related toxicities observed with the growth-factor supported dose-escalated CHOP resembled those observed after high-dose chemotherapy. The German High-Grade Non-Hodgkin’s Lymphoma Study Group (DSHNL) has therefore investigated the dose-escalation of a CHOP-like regimen with etoposide, the CHOEP-regimen29 with stem-cell support. In the Mega-CHOEP regimen, patients receive an initial escalated conventional CHOEP followed by three high-dose CHOEP-regimens with stem cell support. Since the cycles are given at the usual 21-day intervals, total doses of doxorubicin of 280 mg/m², cyclophosphamide >18,000 mg/m² and etoposide >2,000 mg/m² can be administered and achieve a high dose-intensity of the most active cytotoxic drugs for aggressive NHL. Since 1997, 140 patients have been treated in this phase I/II trial, demonstrating the feasibility of the approach. No cases of myelodysplastic syndrome or secondary acute leukaemia have been observed. A phase III trial comparing MEGA-CHOEP to a conventional CHOEP regimen in young high- and high-intermediate risk patients with aggressive lymphoma will be started in late 2000.

While haematopoietic growth factors have been mainly employed to facilitate dose escalations, the DSHNL has pursued a different strategy. Realising that without stem cell support hematopoietic growth factors can add more to dose intensification by shortening treatment intervals than by increasing the dose of one or several cytotoxic drugs, we addressed the question of whether shortening the treatment intervals could improve treatment results.30 This question was tested in two trials, one in which young patients with low LDH were included, and a second trial into which patients >60 years of age, both with normal and elevated pretreatment LDH, were recruited. While the data of these trials involving more than 1,500 patients are far from being complete, an interim analysis of CR rates indicates that shortening the treatment intervals is of benefit to patients with elevated LDH, while for younger patients with normal LDH increasing the dose of cytotoxic drugs may improve CR rates. Whether these trends in CR rates will ultimately translate into better freedom from treatment failure rates or even higher cure rates remains to be shown. However, the different effect of shortening treatment intervals and of increasing total dose for different groups of patients suggests that the two strategies might be successful in patients with aggressive NHL in different prognostic groups.

Immunotherapy

In phase I and phase II trials in patients with follicular lymphomas the chimeric monoclonal anti-CD20 antibody rituximab demonstrated a significant efficacy, i.e. 46% overall response with a very good safety and low toxicity profile.32 Since aggressive B-cell NHL express the same CD20 target antigen on the surface of their neoplastic cells, it was a logical step to study the activity of this antibody in aggressive B-cell lymphomas. An encouraging response rate of 35% was observed when rituximab was given as a single agent in a phase-II trial of patients with untreated and relapsed high-grade NHL.33 In a phase I-II trial of untreated patients with intermediate- or high-grade NHL rituximab was combined with CHOP chemotherapy. Rituximab was given two days before CHOP chemotherapy at 3-week intervals for 6 cycles. In 31 patients, side effects were comparable with those seen with CHOP alone.34 Three large randomised trials comparing the combination of rituximab and CHOP with CHOP chemotherapy alone are currently recruiting patients.

Early results suggest that a radiolabelled CD20 antibody is able to induce responses in patients whose NHL was resistant to the native antibody.35 The role of radioimmunoconjugates in the treatment of aggressive lymphomas, both in a conventional and in a high-dose setting with stem cell support has to be defined in prospective trials. Other immunotherapeutic approaches which have proven their efficacy in indolent lymphomas, e.g. vaccination with immunoglobulin idiotypes or other NHL-associated antigens, have not been tested in a sufficient number of patients with aggressive lymphomas. Apart from the immunglobulin idotype, no other structures that are immunogenic have been shown to be expressed by the neoplastic cells of aggressive B-NHL, i.e. no other structures are recognised by the patient’s immune system which might therefore be used for strategies of active immunotherapy (e.g. the development of specific vaccines).

Mystics, maths and models

The results of the intergroup trial by Fisher et al.12 question the concept of increasing dose intensity to treat aggressive NHL.10,11 The fact that MACOP-B which has nearly double the dose intensity of standard CHOP but is no better than this latter suggests that fundamental assumptions of the dose-intensity concept do not withstand scrutiny. One of the fundamental errors of the hypothesis might be the assumption that all drugs in a regimen are equally effective and that there are no significant differences in terms of the relative contribution of a cytotoxic

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drug to the efficacy of the entire polychemotherapy regimen. In a reconsideration of the Skipper model for chemotherapy, Loeffer and colleagues developed a novel statistical model. In contrast to the Hryniuk model, the effective dose (ED) model takes tumour growth as well as chemotherapy effects including total dose and application intervals into account. The model was used to simulate the effect of various treatment strategies with dose escalations and schedule changes. On the basis of such simulations the authors predicted a gain in therapeutic efficacy of BEACOPP over COPP+ABVD that came close to the one which was later observed when the respective trial of the German Hodgkin’s Study Group was analysed. The ED concept has not only provided us with explanations for the dead-lock in the development of more effective treatment concepts for aggressive NHL in the last two decades, but it may also become a model that helps us to predict the probability of success of new therapeutic strategies and thus might be of value in designing new protocols that will eventually lead to significant progress in the treatment of patients with lymphoma.

References
25. Kaiser U, Uebelacker I, Birkmann J, Havemann K, for the DSNHNL. High dose therapy with autologous stem cell transplantation in aggressive NHL: results of


Relapsed lymphoma

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Patients with Hodgkin’s disease (HD) or aggressive non-Hodgkin’s lymphoma (NHL) can successfully be treated by polychemotherapy plus/minus radiotherapy. Even patients with advanced disease (stage III or IV) frequently achieve a complete remission and one-half to two-thirds of patients who achieve complete remission may remain disease-free. Patients with indolent lymphoma can also achieve complete and partial remissions but in contrast to other lymphoma patients continue to progress or relapse after initial chemotherapy and their survival curves lack a true plateau indicating that they are not ultimately cured. Accordingly, the basis on which to start discussing the clinical management of relapsed lymphoma is different for the different subentities. This article summarises the results of conventional treatment and high-dose therapy (HDT) followed by autologous or allogeneic transplantation of haematopoietic stem cells (HSCT) in the most frequent and well-established disease categories, i.e. indolent (low-grade) NHL, aggressive (high-grade) NHL, and Hodgkin’s disease (HD).

Hodgkin’s disease
Patients with HD who relapse after having achieved first CR can undergo salvage treatment including radiotherapy, conventional salvage chemotherapy, or high-dose chemotherapy (HDCT) with autologous or allogeneic stem cell rescue.

Salvage treatment for relapse after radiation therapy
Patients who relapse following radiation therapy alone for localised HD (stage I and II) have satisfactory results with combination chemotherapy. Stage at relapse is an important prognostic variable when radiation fails to cure the disease. A study from Stanford showed that conventional salvage chemotherapy was sufficient in patients with limited stages who relapsed following (sub)total nodal irradiation and had no systemic symptoms at the time of recurrence. After 10 years, 90% of these patients remained disease-free. In contrast, the 10-year disease-free survival (DFS) for those with stage III or IV disease or with B symptoms at the time of relapse was 58% and 34%, respectively.1

Salvage chemotherapy for relapse after combination chemotherapy
In 1992 the National Cancer Institute (NCI) reported their experience with the long-term follow-up of patients who relapsed after MOPP.7 Chemotherapy failures were divided into three subgroups: 1) primary progressive disease, 2) early relapse within 12 months of CR, and 3) late relapse after CR lasting >12 months. For patients with primary refractory disease, the projected 8-year survival was 0. For patients with early or late relapse the projected 20-year survival was 11% and 22%, respectively. Fewer than 30% of patients relapsing after a short initial remission achieve a second CR even when treated with non-cross-resistant regimens and have a median survival of 2.5 years. In contrast, more than 80% of patients with late relapse achieve a second CR with MOPP or alternative regimens and have a median survival of >4 years.3 Other conventional salvage regimens show similar clinical outcomes, with fewer than 30% of the patients remaining disease free in the long-term.4-8

Radiation therapy for relapse after combination chemotherapy
Selected patients who relapse after chemotherapy in an isolated nodal site without systemic symptoms can be salvaged by radiotherapy alone. Failure-free survival and overall survival can be expected in 30% and 50% of patients, respectively, if adverse prognostic factors such as B-symptoms, involvement of extranodal sites, and older age are absent.9,10

High-dose chemotherapy and autologous haematopoietic stem cell transplants
HDCT followed by HSCT has been shown to produce 30-65% long-term DFS in selected patients with refractory and relapsed HD.12,13 The most compelling evidence for the superiority of HDCT in relapsed HD comes from reports from the British National Lymphoma Investigation (BNLI) and the GHSG together with the European Group for Blood and Marrow Transplantation (EBMT). In the BNLI trial, patients with relapsed or refractory HD were treated with a combination of carmustine (BCNU), etoposide, cytarabine and melphalan at a conventional-dose level (mini-BEAM) or a high-dose regimen (BEAM) with autologous bone marrow transplantation. The actuarial 3-year EFS was significantly better in patients who received HDCT (53% vs. 10%).14 The largest randomised multicentre trial was performed by the GHSG/EBMT.15 Patients with relapse after chemotherapy were randomly assigned to either four cycles of DEXA-BEAM...
Currently, a number of salvage approaches for patients with relapsed non-Hodgkin’s lymphoma are being developed. For instance, one therapy involves the use of high-dose chemotherapy with autologous bone marrow or blood stem cell rescue. This approach has been shown to achieve response rates of approximately 50% in patients with chemosensitive disease. However, the integration of new drugs into combination chemotherapy protocols has just begun and it remains to be settled whether better survival rates can be achieved.

High-dose therapy and autologous blood or marrow transplantation

The disappointing results of conventional dose chemotherapy in patients with relapsed aggressive lymphoma led to the early and frequent use of high-dose therapy followed by transplantation of autologous bone marrow or peripheral blood progenitor cell transplantation in this setting. Numerous phase II studies of high-dose therapy (HDT) and transplantation of autologous HSCs for relapsed NHL have shown that this approach may benefit from HDT, with reported long-term survival rates between 40% and 60%. However, the integration of new drugs into combination chemotherapy protocols has just begun and it remains to be settled whether better survival rates can be achieved.

Aggressive non-Hodgkin’s lymphoma

Current first-line chemotherapy protocols in some instances followed by involved-field radiotherapy result in long-term disease-free survival of 25–35% of patients with aggressive lymphoma depending on the risk profile of the individual patient (IPI-score). All other patients will need further therapy for relapse or primary progressive disease.

Conventional dose salvage therapy for patients with aggressive NHL includes a wide variety of drugs, alone or in combination, depending on the type of lymphoma. However, the effectiveness of these therapies is limited, and many patients will require further therapy.

It is important to note that the choice of salvage therapy is based on the patients' risk profile, including factors such as age, performance status, and the presence or absence of bulky disease. The response rates for salvage therapy are generally lower than for first-line therapy, and the outcomes are more heterogeneous. However, the integration of new drugs into combination chemotherapy protocols has just begun and it remains to be settled whether better survival rates can be achieved.

Further analyses of the PARM A study as well as the results of various retrospective studies suggest that...
the success of high-dose therapy and autologous HSC transplantation. The failure of HCT depends largely on chemosensitivity of the disease (see above) but also on the time interval between first-line therapy and relapse. Patients with primary progressive disease and a short interval between first-line therapy and relapse also do poorly with high-dose therapy. An analysis of the PARMA study showed that patients with a remission duration of less than 12 months had a progression-free survival of 8% in contrast to those patients with a remission duration of > 12 months who had a PFS of 20%. Overall survival was 13% in patients with short remission duration as opposed to 29% in patients with longer remission duration. Very similar to its importance in first-line therapy the International Prognostic Index (IPI) is also significantly correlated with the probability of progression-free survival after high-dose therapy.

There is no convincing evidence that use of other drugs, tandem transplants or sequential high-dose therapy regimens give results superior to “classical” high-dose therapy. Likewise, different sources of haematopoietic stem cells (BM or blood), the administration of haematopoietic growth factors after BMT or PBPC, or any other change in supportive care have not yet been convincingly shown to improve treatment outcome.

Allogeneic blood or bone marrow transplantation

Allogeneic blood and marrow transplantation have not frequently been used in aggressive lymphoma, the reason being that the high transplant-related mortality outweighed the lower relapse rates seen after allogeneic transplantation. DFS or overall survival after allogeneic blood or marrow transplantation was similar or inferior to that after autologous transplantation regardless of the histological subtype or the disease status of the patient. This may change with new strategies using reduced conditioning with fludarabine-based pre-paratory regimens accompanied by vigorous GVHD prophylaxis and transplantation of bone marrow or blood stem cells. For the first time this approach may allow patients to enjoy the benefits of the graft-versus-lymphoma effect but avoid the toxicity of myeloablative regimens. So far, the number of patients treated with so-called mini-allografts is too small, treatment modalities have been too heterogeneous, and follow-up too short to evaluate fully the importance of this new modality. Early experience suggests, however, that patients with aggressive lymphoma may be less responsive to mini-allografting than patients with indolent lymphoma.

Indolent lymphoma

Indolent lymphomas are heterogeneous disorders which usually respond to single- or multiagent chemotherapy; frequency the completeness of response depend largely on the disease stage and histological subtype but also on the type of chemotherapy administered to the patient. Single agents such as chlorambucil rarely induce more than partial remissions while the new nucleoside analogues (fludarabine, cladribine) are able to induce partial and complete remissions in up to 30% of patients depending on the extent of prior therapy. Combination chemotherapy programmes including nucleoside analogues, for instance the FCM regimen (fludarabine, cyclophosphamide, and mitoxantrone) are among the most effective treatment modalities available today and can result in complete remission rates of up to 50% even in heavily pre-treated patients including molecular remissions in some of the patients such treated. Unfortunately, patients not achieving a complete remission with first-line therapy will progress again and virtually all patients with chemotherapy-induced CR will relapse within months if treatment is discontinued. The chimeric human-mouse monoclonal anti-CD20 antibody (rituximab) represents another type of new effective treatment which has raised considerable interest during recent years. Mc Laughlin et al. reported that half of 151 assessable patients treated for multiply relapsed low-grade NHL responded to an infusion of the antibody with partial or complete remission; the projected median time to progression was 13 months. Prognostic factors indicating a favourable response to the antibody were positivity for the bcl-2 gene rearrangement at baseline, response to last chemotherapy, and follicular small cleaved, follicular mixed, or follicular large cell versus small lymphocytic histology. Current clinical trials have been designed to compare the effects of aggressive chemotherapy programs (e.g., FCM) alone with the same regimen given in conjunction with rituximab. In some studies rituximab will also be administered as maintenance therapy. No results of such trials are currently available.

High-dose therapy and transplantation of autologous haematopoietic stem cells

HDT followed by transplantation of autologous HSCs has been used to treat relapsed indolent lymphomas. Again, chemosensitive patients do better than patients with chemoresistant disease; however, in contrast to Hodgkin’s disease or aggressive lymphomas, patients transplanted for indolent disease continue to relapse for many years after HDT. The comparison of HDT/HSCT in second remission of indolent lymphoma with a historical control group suggests that freedom-from-recurrence may be prolonged by high-dose therapy. No prospective randomised trials have been reported in order to answer this question definitively. Several phase II studies have suggested that purging of bone marrow or peripheral blood progenitor cells with monoclonal antibody may improve treatment outcome; phase III studies directly comparing the outcome of transplantation using purged versus non-purged marrow or peripheral blood progenitor cells are not available. The EBMT is currently conducting a trial comparing the use of unpurged cells versus peripheral blood stem cells purged in vivo by virtue of rituximab i.v. followed by BEAM chemotherapy. Patients in one arm of the study will also receive rituximab for maintenance therapy.

Different strategies have been implemented to label anti-CD20 or anti-CD22 antibodies with iodine 131 or yttrium 90 in order to increase the effectiveness of monoclonal antibody targeted therapy. Preliminary results of these and other trials look promising and deserve further study.
Allogeneic transplantation
As with the other lymphoma entities allogeneic bone marrow transplants have rarely been performed in patients with low-grade non-Hodgkin's lymphomas. Although the relapse rates after allogeneic BMT/PBPCT are lower than after autologous transplantation the high transplant-related mortality outweighs this potential benefit and disease-free and overall survival after autologous or allogeneic transplantation in patients with low-grade lymphoma have overlapped.

The situation may change if preparatory regimens using reduced conditioning are used. Preliminary data from various institutions indicate that non-myeloablative conditioning followed by transplantation with allogeneic bone marrow or peripheral blood progenitor cells may be particularly successful in patients with indolent lymphoma and chronic lymphocytic leukaemia. These data need further confirmation within prospective, multi-institutional trials before this strategy is used more frequently.

References
Session 2 – HIGH GRADE NON-HODGKIN’S LYMPHOMA


The molecular pathogenesis of high grade non-Hodgkin’s lymphoma

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Classification of non-Hodgkin’s lymphomas (NHL) has historically been based on histological and immunological criteria. However, even in the best hands, this is not adequate! Malignancy is a genetic disorder, with abnormalities of key genes associated with specific subtypes of disease and responses to therapy, as is best illustrated by the acute leukemias (AL). Compared to AL, genetic classification of NHL is less advanced. Cytogenetic studies in high-grade NHL have been hampered by difficulties in obtaining fresh tumour cells, but also by histological diversity and cytogenetic complexity. Whilst AL characteristically exhibit only one chromosomal translocation, NHL often exhibit complex cytogenetic abnormalities, reflecting the necessity for concurrent activation of dominant oncogenes and inactivation of tumour suppressor genes. The biological behaviour of the NHL is therefore less likely to be reflected by a single genetic abnormality, but rather by the sum of genetic damage within the neoplastic clone. This is particularly true in the high-grade NHL, which may arise as a consequence of transformation of indolent disease. Several markers may need to be analysed in order to predict outcome. In this paper, we review the genetic changes in high-grade NHL according to histological subtype and summarise their clinical impact.

The search for genetic changes in malignancy has traditionally been led by cytogenetic analysis and remains the focus for much of this paper. However, new techniques, such as gene profiling, allow the isolation of new diagnostic markers. This has been shown recently in the case of high-grade NHL (ref. #1 and http://llmpp.nih.gov/lymphoma).

General considerations

Tumour tissue containing viable cells is mandatory for chromosome analyses. For global detection of genetic changes within a clone, cytogenetics remains the gold standard. But, even in the best hands conventional karyotyping is inadequate to clarify the origin of chromosomal markers in complex karyotypes! Thus, cytogenetics should be supplemented by modern fluorescence in situ hybridisation (FISH) techniques such as multi-colour spectral karyotyping (SKY) using chromosome painting or single copy probes to confirm assignments. SKY has not been used systematically in high-grade NHL, but has revealed previously undetected translocations in myeloma.2 Like conventional chromosome analyses, SKY is also hampered by the necessity of tumour metaphases. This limitation of cytogenetics and SKY can be overcome by interphase FISH, which can detect recurrent genetic alterations even if no metaphases can be obtained, as it can be used in cryopreserved specimens or smears. Interphase FISH analyses are indicated whenever lack, low quantity or low quality of tumour metaphases prevents complete karyotyping or when only normal metaphases are obtained.

Some subgroups of high-grade NHL are characterised by specific chromosomal translocations usually involving the immunoglobulin (IG) loci and some of these translocations are specific for a subgroup of disease indicating a potential diagnostic use (Table 1). The IG translocations can be detected by various methods. All IGH breakpoints at 14q32 by FISH can be screened for by using two differently labelled probes hybridising to each side of the breakpoint. Cosmid probes for the IGH constant and variable regions are often used. Cells with two normal chromosomes 14 display two-colour signals for the intact IGH locus, whilst IGH translocations lead to dissociation of one two-colour signal, which is detectable in interphase cells.2 Preliminary FISH studies using this approach suggest that IGH translocations, as for example translocations involving subtelomeric regions like t(4;14)(p16;q32) or t(14;16) (q32;q23) typical for multiple myeloma, are more frequent than assumed. Such FISH methods can be extremely sensitive, capable of detecting only minor sub-clones which may nevertheless be of biological importance in the progression of the disease.3 Problems remain however, in performing FISH routinely on paraffin sections. Fibre-FISH performed on extended DNA fibres may also be of value, although this is a difficult technique to perform.

Alternatively, since most of the IG breakpoints are clustered, it has been possible to design polymerase chain reaction (PCR) methods to amplify from these known sequences across the translocation breakpoint, using long-distance inverse PCR.3 This method is simple and robust. It does, however, require high molecular weight DNA and has not yet been applied systematically to high grade NHL. The principal consequence of IG translocations is deregulated expression of the incoming oncogene. In some instances, such as BCL2, it is possible to detect the over-expressed protein using M Abs. However, BCL2 over-expression may arise as a result of mechanisms other than translocation, including BCL2 promoter demethylation and gene amplification and thus, over-expression and chromosomal translocation do not
correlate. MYC may not be over-expressed as a result of translocation, but normal control mechanisms that limit expression specifically to the early G1 phase of the cell cycle are lost resulting in constitutive expression throughout the cell cycle. To complicate matters further, it is now clear that at least in some instances genes on both derivative chromosomes may be deregulated by IG translocations. The consequences of this remain to be determined.

Most IG translocations probably arise in mature B-cells, as early or primary events in the course of the disease. Mouse gene inactivation studies have indicated that defects in DNA repair genes predispose to their formation. However, it is clear that on their own, IG translocations do not result in transformation. This has been shown in transgenic mouse experiments in which the oncogene is expressed under the control of the IG locus, recapitulating the effects of the IG translocation. Also, apparently normal B-cells with IG translocations can be found in mice and man. These data emphasise the importance of secondary genetic events in the development of the neoplastic phenotype. How the association of specific translocations with specific subtypes of disease is mediated is not known.

Like all malignancies, high-grade NHL also exhibit recurrent deletions indicative of inactivation of tumour suppressor genes and genomic amplifications indicative of activation of oncogenes. The molecular consequences of many of the common deletions remain to be determined. With the exception of Burkitt’s lymphoma, mutations of p53 are secondary events and are common in transformed and progressive disease and associated with a poor prognosis. Deletion of the p16/p19ARF locus on chromosome 9p21 has been associated with transformation to high-grade disease as have BCL2 mutations. Many of the latter mutations cluster in the NH2-terminal domain, which normally mediates a cell cycle inhibitory effect. Down-regulation of other important cell-cycle regu-

<table>
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<tr>
<th>Chromosomal Translocation</th>
<th>Involved Oncogene</th>
<th>Disease/Frequency</th>
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<tbody>
<tr>
<td>t(8;14)(q24;q32) and variants</td>
<td>MYC</td>
<td>Burkitt’s -100% transformed follicular NHL - 10%</td>
</tr>
<tr>
<td>t(14;18)(q21;q21) and variants</td>
<td>BCL2</td>
<td>Centroblastic DLBCL - 30%</td>
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<tr>
<td>t(3;14)(q27;p32) and multiple variants including non-IG partner genes</td>
<td>BCL6</td>
<td>Centroblastic DLBCL - 20%</td>
</tr>
<tr>
<td>t(11;14)(q13;p32)</td>
<td>BCL1/CCND1; Cyclin D1</td>
<td>Blastic transformation of mantle cell NHL - 100%</td>
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<tr>
<td>t(2;5)(p23;q35)</td>
<td>NPM-ALK</td>
<td>Anaplastic large cell lymphoma</td>
</tr>
<tr>
<td>inv(14)(p21;q32)</td>
<td>ATC-ALK</td>
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<tr>
<td>t(1;2)(q25;p23)</td>
<td>TPM3-ALK</td>
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<tr>
<td>t(2;3)(p23;q21)</td>
<td>TFG-ALK</td>
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Sporadic, but recurrent IG translocations in DLBCL

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<th>Chromosomal Translocation</th>
<th>Involved Oncogene</th>
<th>Disease/Frequency</th>
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<tbody>
<tr>
<td>t(11;14)(q21;q32)</td>
<td>BCL11</td>
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<tr>
<td>t(11;14)(q21;q32)</td>
<td>BCL11</td>
<td></td>
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<tr>
<td>t(12;14)(q11;p32)</td>
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</tr>
<tr>
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<td>Unknown</td>
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</tr>
<tr>
<td>t(12;14)(q13;q32)</td>
<td>BCL1A</td>
<td></td>
</tr>
<tr>
<td>t(14;15)(q23;p11;q13)</td>
<td>?BCL8</td>
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</tr>
<tr>
<td>t(X;14)(p11;q32)</td>
<td>Unknown</td>
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These data have in part been adapted from Cigudosa et al., 1999, a purely cytogenetic study of 215 successfully karyotyped DLBCL. Of these 110 exhibited IG translocations, 42 involving BCL2, 21, MYC and 14 BCL6, leaving 33 falling within uncloned loci. All of these occurred singly with the exception of t(9;14) (p13;q32), which was observed in 4 cases; whether this translocation involves the PAX5 gene as seen in low-grade lymphoplasmacytoid lymphomas with a cytogenetically identical translocation is not known. Note that t(11;14)(q21;21) can involve at least 3 different partner genes within chromosome 1q21, depending on the cell type in which the translocation arises. All the “sporadic” translocations are recurrent, non-random events. Whether they have any clinical significance in the context of DLBCL is not known. A comparable broad spectrum of IG translocations is also seen in myeloma. A full list of references covering both the cytogenetic descriptions of all the above abnormalities and their molecular cloning may be found in ref. #16.
Burkitt's lymphoma – MYC/IG translocations

Burkitt's lymphoma is curable with intensive combination chemotherapy. It is, therefore, essential that there is an objective method for diagnosing this disease. MYC/IG translocations are found in nearly all cases of Burkitt's lymphoma but also sporadically in other high-grade B-cell NHL; the latter group often co-express t(14;18)(q32;q21) and also have a poor prognosis. The genetic problem is that the breakpoints in the MYC locus on 8q24 are not clustered but can fall up to 500 kb centromeric or telomeric of the MYC gene itself.

Cytogenetics: the translocation t(8;14)(q24;q32) or its variants (t(2;8)(p13;q24) and t(8;22)(q24;q11) are found in most, if not all, Burkitt's lymphomas and sporadically, in other high-grade B-cell lymphomas. Recurrent secondary changes comprise duplications in 1q, deletions in 6q, trisomies 7 and 12 and structural changes in 13q. The molecular consequences of these secondary changes are not known.

FISH-assay for the detection of t(8;14)(q24;q32): different FISH approaches for the detection of the t(8;14)(q24;q32) have been established using either probes hybridizing to both sides of the MYC breakpoint in 8q24 alone, or combining them with probes for IGH. In the t(8;14)(q24;q32), the 8q24 breakpoints map centromeric to MYC, whereas those for the variant translocations cluster telomeric to MYC. Most of the assays are only suitable for the detection of the classical t(8;14). Nevertheless, using probes telomeric to MYC, the variant translocations have been detected.

Molecular consequences: despite the diversity of 8q24 breaks, MYC seems to be the target gene in all instances. MYC over-expression on its own can result in either apoptosis or increased proliferation depending upon the genetic background of the cell. The apoptotic function is blocked in Burkitt's lymphomas (in which detectable BCL2 is often lacking) by p53 mutations and in transformed follicular B-NHL by BCL2 over-expression, resulting in enhanced proliferative drive. How MYC mediates these effects is unclear. MYC transgenic mice accumulate large populations of undifferentiated pre-B lymphocytes and eventually develop lymphoma by sustaining spontaneous inactivation of either p53 or other genes on the same pathway demonstrating that inactivation of this pathway is an important step in MYC-mediated oncogenesis. The involvement of genes able to induce both apoptosis and proliferation is a recurrent theme in lymphomagenesis and includes FcYRIIB, BCL6 and BCL10.

Diffuse large B-cell lymphomas (DLBCL)

DLBCL comprise 30-40% of all NHL. This histological subgroup probably contains several separate entities and there is debate whether DLBCL should be subdivided into different histological subtypes, namely centroblastic (CB) and immunoblastic (IB). There are now both cytogenetic and gene profiling data supporting this subdivision. Recurrent cytogenetic translocations and in transformed follicular B-NHL by BCL2 over-expression, resulting in enhanced proliferative drive. How MYC mediates these effects is unclear. MYC transgenic mice accumulate large populations of undifferentiated pre-B lymphocytes and eventually develop lymphoma by sustaining spontaneous inactivation of either p53 or other genes on the same pathway demonstrating that inactivation of this pathway is an important step in MYC-mediated oncogenesis. The involvement of genes able to induce both apoptosis and proliferation is a recurrent theme in lymphomagenesis and includes FcYRIIB, BCL6 and BCL10.

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11q22-23 involving the DNA repair gene ATM, deletions in 1p, deletions in 9p involving p16/p19ARF, deletions of chromosome numbers in the tetraploid range. Deletions in high-grade mantle cell lymphomas often have chromosome t(11;14)(q13;q32), can be detected cytogenetically. The high-grade blastic or blastoid variants with a poor prognosis, but this is particularly so for the patients with CB lymphomas.

FISH-assay for the detection of the translocation t(11;14) (q13;q32): different single- and double-colour FISH assays have been applied for the molecular cytogenetic detection of t(11;14) in interphase cells. In a single-colour FISH approach a t(11;14)(q13;q32) was indicated by split of a YAC-DNA probe spanning the BCL1-locus. Nevertheless, for reasons that are not clear, BCL1-containing YACs have been found to be highly unstable. Furthermore, the detection of split signals of a BCL1 YAC may be unreliable, as the part of the YAC remaining on the aberrant chromosome 11 may be too tiny and, thus, difficult to detect. Thus, double-colour FISH assays have been developed using probes flanking the 11q13 breakpoint or combining an IGH and a BCL1 probe. As with t(14;18)(q32;q21), these assays seem to be superior to cytogenetics and molecular techniques at the time of diagnosis. Fibre-FISH assays have been developed to visualise the t(11;14)(q13;q32) (4).

Molecular consequences: this translocation results in deregulated expression of the cyclin D1 gene. In NHL cell lines carrying the t(11;14)(q13;q32) translocation, although cyclin D1 is still regulated in its normal cyclical manner (unlike MYC in Burkitt’s lymphomas), its over-expression causes acceleration through G1 phase. However, although cyclin D1 over-expression is sufficient to confer transformed properties to established fibroblasts, it is insufficient to transform primary cells or to induce lymphomagenesis in Em-cyclin D1 transgenic mice. These mice demonstrated remarkably few abnormalities in their lymphoid population. Consistent with a multi-step process of tumourigenesis, however, cyclin D1 collaborates strongly with MYC in lymphomagenesis in double transgenic animals.

Extranodal and mediastinal B-cell lymphomas.

Extranodal NHL comprise a large and highly heterogeneous subgroup of diseases. Cytogenetic data on extranodal high-grade lymphomas and on primary mediastinal B-cell lymphomas are rare. Although not associated with a certain subtype of high-grade B-cell NHL, t(3;14)(q27;q32) or variant translocations affecting BCL6 at 3q27 have been reported to be particularly frequent in high-grade B-cell lymphoma with extranodal involvement. Subsets of primary extranodal high-grade B-cell lymphomas of MAL-T-type contain the t(1;14)(p22;q32) involving BCL10, trisomies 3 and 7 as well as t(8;14)(q24;q32) and other changes affecting the MYC locus at chromosome 8q24. In marginal zone lymphomas, gains of 1q and 8q and losses of chromosome 17 or 17p occurred more frequently in relapsed or progressive lymphomas; high-level amplifications were seen in 8q. CGH-studies in large B-cell lymphomas of the gastrointestinal tract additionally pointed to gains of chromosomes 11, 12 and 1q and to several high-level amplifications, e.g. in 2p13 and 8q24 involving
REL/BCL11 and MYC, respectively. Moreover, according to CGH, mediastinal B-cell lymphomas are characterised by gains of 9p and amplifications of 2p13 again affecting REL/BCL11. The MAL gene, which encodes an interesting cell surface protein, has recently been found to be expressed selectively at high levels in a high proportion of mediastinal B-NHL. The possible clinical value of this observation remains to be determined.

Anaplastic large cell lymphomas (ALCL)

These NHL typically present in younger patients, have a T- or null-cell phenotype with co-expression of CD30, and a good response to therapy, although unexpected molecular heterogeneity is also emerging in this disease subset. The role of the t(2;5)(p23;q35) in this disease has been reviewed. This translocation is similar to those seen in AL with fusion transcript of the two genes, ALK on chromosome 2p23 and NPM on 5q35, being produced thus allowing detection by RT-PCR.

Cytogenetics: t(2;5)(p23;q35) is characteristic of ALCL of T-cell or null-type, although variants are now being identified.

FISH assay: one approach used probes immediately centromeric to NPM and an ALK probe located telomeric to the 2p23 breakpoint so that the t(2;5) (p23;q35) was seen by co-localisation of both signals. The recent introduction of an assay using probes flanking the ALK-locus revealed unexpected insights into the pathogenesis of CD30-positive anaplastic large cell lymphomas. By means of this FISH system, previously unknown variant rearrangements such as the cryptic inv(2)(p23q35) or t(1;2) (q25;p23) were identified. These FISH assays allowed the molecular characterisation of the resulting fusion genes, ATIC-ALK and TPM3-ALK respectively. The key event in these translocations is the ectopic expression of ALK, which can be readily detected by immunohistochemistry. In two studies, ALK+ve ALCL had a better prognosis than those lacking ALK expression and in one, a subset of patients with only cytoplasmic but not nuclear and cytoplasmic ALK was identified. Patients with only cytoplasmic ALK represented patients with variant translocations.

Conclusions

With the increasing use of high-dose chemotherapy, there is an urgent need to identify prospectively those patients with high-grade NHL destined to relapse following conventional therapy, who might possibly be cured by additional therapy in first remission. It is unlikely, in most subsets of disease, that a single marker will be sufficiently discriminatory. The application of whole-cell approaches, such as gene profiling and proteomic analysis, along with the availability of the complete human genome sequence, will allow the rapid identification of a plethora of new markers of diagnostic, and prognostic potential. The challenge will be to incorporate these markers into routine clinical practice in a cheap and simple fashion, even on formalin-fixed material! Further characterisation of the pathways by which these proteins mediate transformation will allow the development of new therapeutic approaches.

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References


**New insights into myeloma biology**

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Development and composition of the myeloma tumor clone

During disease evolution, bone marrow plasma cells are the predominant cell type in multiple myeloma (MM). However, several studies have indicated that the tumour clone also includes circulating B-cells that might represent the myeloma stem cell. Clear evidence supporting this concept was provided by molecular techniques showing that MM patients have circulating B-cells that harbour the same immunoglobulin (Ig) gene rearrangements as the malignant bone marrow plasma cells. Moreover it was demonstrated that these circulating myeloma cells have somatic mutations in their Ig genes without intrachromosomal variation. This implies that MM originates from a B-cell that has been antigen-selected in the germinal centre of a lymph node. On the other hand these molecular techniques do not give more precise information about the real differentiation stage of this circulating myeloma precursor cell. According to the homing features of normal B-cells, it might theoretically be a plasmablast or a CD19 expressing B-lymphocyte. Immunophenotypic analyses indicated that in early-diagnosed myeloma patients as well as in normal individuals very small amounts of plasma/macritic cells are also detectable in the peripheral blood. These circulating cells seem to reflect a more immature phenotype (CD45+, VLA-5–) than the bone marrow derived plasma cells (CD45+, VLA-5+) but in MM patients express the same (monoclonal) Ig isotype. Several studies also indicated that patients with active disease have more circulating monoclonal plasma cells than those with inactive disease. Therefore, the number of circulating plasma cells has also been identified as a reliable predictor of survival in MM patients. Based on all these data it can be assumed that the bulk of the tumour consists of non-proliferating and fully matured plasma cells which are fed by a pool of more immature precursor cells which constitute the proliferative compartment and determine the clinical behaviour of the patient.

Although the oncogenic transformation process remains poorly understood, there is much evidence suggesting that chromosomal translocations involving the immunoglobulin heavy chain (IgH) gene are an important initiating event in the pathogenesis of MM. These translocations usually occur into Ig switch regions and involve three non-random loci that are related to cell proliferation, i.e. 11q13 (bcl-1, cyclin D1), 16q23 (c-maf) and 4p15 (FGFR3/M SET). The juxtaposition of the oncogenes to powerful Ig regulatory elements leads to an ectopic expression in the myeloma cells. The fact that these specific chromosomal anomalies can also be found in patients with monoclonal gammapathies of undetermined significance (MGUS) makes it likely that they are related to the very early cell immortalisation. Since approximately 25% of MGUS will develop MM, it can be assumed that this malignant progression is caused by the occurrence of additional genetic defects. Indeed, very sensitive molecular techniques revealed the presence of different karyotypic defects in most, if not all myeloma patients studied. The upregulation of IL-1β expression in plasma cells as well as the occurrence of monosomy 13 have recently been identified as two more specific factors that might contribute to the transition from MGUS to MM.

**Homing and growth features of myeloma cells in the early phase of disease**

Selective homing of myeloma cells to the bone marrow

The restricted localisation of myeloma cells in the bone marrow (BM), during the initial stage of the disease could be explained by a selective initial homing of the circulating myeloma cells to the bone marrow microenvironment and/or by the presence of a unique local microenvironment supporting survival and growth of the myeloma cells. The initial homing can be defined as the set of molecular interactions that allow circulating cells to recognise, adhere to and migrate across bone marrow endothelial cells and results in the accumulation of myeloma cells in the bone marrow microenvironment which in turn can support proliferation of the tumour cells. To answer the question of whether the restricted localisation of myeloma cells in the bone marrow is the result of selective migration towards the bone marrow and/or selective survival in the bone marrow, we performed a study in the murine ST M model. This model was initially developed by Radl in C57BL/ KaLwRij mice. It includes several cell lines of which the 5T2 line most closely resembles human MM in several aspects: the course of the disease is moderately progressive, the homing is more restricted to the BM, the level of serum paraprotein is related to disease progression and the development of osteolytic lesions can consistently be observed during the development of the disease. Transplantation of the bone marrow of these MM-carrying mice by intravenous injection into young syngeneic recipients led to a reproducible continuous in vivo propagation of the MM malignancy. In order to distinguish a selective homing of
5T2MM to the bone marrow from a more random process to a number of tissues it was essential to determine accurately the organs infiltrated by the MM cells after intravenous injection. Therefore MM cells were isolated out of diseased animals, labelled with $^{51}$Cr, injected intravenously and then the radioactivity was traced at different times after injection into different organs. When a clear-cut serum paraprotein concentration was observed in all mice, the organ distribution of the 5T2MM cells was analysed using a combination of histology and immunochemistry. The growth of the 5T2MM cells was found to be restricted to the BM in all mice with half of the mice having a limited infiltration in the spleen. A very low percentage of the mice showed liver infiltration. Moreover we demonstrated that the restricted localisation of 5T2MM cells in the BM and spleen, as observed in diseased mice could be explained by selective entry of the MM cells into BM, spleen and liver and by the presence of a unique local microenvironment in BM (and partly in the spleen) but not in the liver.10

Migration and extravasation of myeloma cells

The fact that small amounts of myeloma cells are detectable in the peripheral blood and that their number increases with disease progression, makes it very likely that these circulating cells represent the component of the tumour clone that mediates disease dissemination. This implies that these cells must have the potential to extravasate and home to the bone marrow environment. In analogy to the migration mechanisms used by normal leukocytes and/or metastatic tumour cells of a non-hematopoietic origin, it can be assumed that this bone marrow homing process is mediated by adhesive interactions and chemotactic signals provided by the tumour-microenvironment. Three groups of molecules are thought to be the key mediators of this homing process, i.e. adhesion molecules, chemotactic factors and metalloproteinases. Migration of leukocytes through the blood circulation into different tissues is a basic feature of normal blood cell physiology. Since myeloma cells are derived from normal B-cells, it can be assumed that they have an important part of their migration and homing programme in common with their normal counterparts. According to the model recently proposed by Butcher and Picker, extravasation of lymphocytes can be divided into four sequential steps.11 In the first step, a primary transient adhesion occurs through interaction between functional lymphocyte receptors and vascular ligands. This interaction results in a rolling of the lymphocyte along the vessel wall. Next, lymphocytes show a reversible, integrin-dependent arrest. Finally lymphocytes pass through the endothelial cell layer by diapedesis. The migration of lymphocytes is triggered by locally produced factors with chemotactic and/or chemokinetic potential. Some of these factors can induce a migration-promoting effect during crossing of the basement membrane while others might stimulate further migration in the interstitial matrix.

The first step of the cascade of homing to a particular organ is the adhesion of the MM cells to tissue specific endothelium. In the 5T2 MM mouse model, we demonstrated that myeloma cells bind se-

Figure 1. An oncogenic transformed post-germinal B cell enters the bone marrow as a malignant plasmablast that interacts with the bone marrow environment to proliferate and differentiate to a fully matured plasma cell. The malignant plasmablasts can re-circulate and re-enter the bone marrow at longer distance. As a result of spilling over also mature plasma cells can rarely be detectable in the circulation.
Haematopoietic stem cells, it can be assumed that close cellular contact between the stroma and the tumour cells raises the effective concentration of locally produced factors, thereby potentiating their biological action. We demonstrated that bone marrow plasma cells express several adhesion molecules, including ICAM-1, VLA-4, N-CAM and CD44, of which the natural ligands can be found in the surrounding marrow stroma. By functional assays we demonstrated that human myeloma cell lines can bind strongly to fibronectin using the VLA-4 receptor, while binding to other extracellular matrix molecules (collagen-1, laminin and hyaluronic acid) was weak or absent. Moreover, we found that myeloma cell lines are able to produce fibronectin themselves. The adhesion of myeloma cells to intact stroma was only partially dependent on fibronectin and its receptors, indicating the involvement of other, perhaps multiple, adhesive mechanisms.

Recently, DNA of Kaposi’s sarcoma-associated herpesvirus (KSHV) or human herpes virus-8 (HHV-8) was found in bone marrow- and blood-derived dendritic cells in MM patients. A causal association between the viral infection and development of MM was suggested, especially because HHV-8 produces a viral IL-6 which stimulates the proliferation of human myeloma cells. However, several groups could not confirm this observation and therefore this hypothesis remains controversial.

Within the bone marrow environment, myeloma cells do not only communicate with stromal fibroblasts but mediate also bone remodelling by production of osteoclast-activating factors (such as IL-1p), inhibition of osteoblasts and activate secretion of bone-degrading proteases (e.g. MMP-9). A recent observation of potential major importance is the occurrence of neovascularisation in MM bone marrow.

It has been demonstrated that angiogenesis parallels progression of MM and that low microvessel density is a favourable prognostic feature. Recent trials reveal a clinical response in MM patients treated with the anti-angiogenesis drug thalidomide. Although the molecular mechanisms that mediate neovascularisation are not yet unravelled, we demonstrated that human myeloma cell lines have angiogenic potential as measured by the chorioallantoic membrane (CAM) assay. Moreover, we and others found that human myeloma cell lines can produce factors that trigger endothelial cells (VEGF, bFGF) and may play an important role in the pathogenesis of this disease.

Homing and growth features of myeloma cells in the late phase of disease

During the end stage of the disease increasing numbers of myeloma cells can be detected in the blood circulation. Moreover, in this phase of disease some patients show tumour localisation at different extramedullary sites such as lung, liver, ascites and pleural fluid. Molecular evolution towards stroma-independency in association with an enhanced extravasation potential are likely to be the key mechanisms that underlie this disturbed homing behaviour. The restricted localisation of myeloma cells to the bone marrow during the initial phase of the disease is most probably related to the local presence of a unique combination of survival- and growth factors, which is not present at other tissue sites. Evolution towards stroma-independency implies that the tumour cells develop molecular mechanisms that result in prevention of apoptosis and autocrine growth stimulation. Another possible mechanism that might help tumour cells to survive and grow outside the bone marrow environment might be reflected by the capacity to produce ECM proteins themselves. Indeed, we observed that myeloma cells can produce fibronectin and osteosialoprotein, two important components of the narrow stromal matrix.

During disease evolution an increasing number of genetic abnormalities can be observed, including mutations in oncogenes (N-ras, c-myc, bcl-2) and deletions in tumour suppressor genes (p53, Rb). So far it is not clear at which level the observed genetic abnormalities correlate directly with stroma-independency and/or enhanced extravasation potential of the tumour cells.

Loss of expression of adhesion molecules has often been postulated to play a role in dissemination of myeloma cells out of bone marrow. Unlike bone marrow plasma cells, extramedullary myeloma cells often show downregulation of LFA-3 or N-CAM. In addition, it was found that myeloma cells have a tendency to lose LFA-1 and VLA-5 when extramedullary spread occurs. Comparing the spontaneous migration capacity of both variants of the MM5 human myeloma cell line, we found that the stroma-independent MM5.2 cells are more motile than the stroma-dependent MM5.1 cells.

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endothelial migration is stimulated by a more specific membrane of the endothelium. Next, the transcellular migration is triggered by laminin-1, present in the basement. In the first instance the motility of the tumour cells is assumed to depend on the functional involvement of adhesion molecules, chemotactic factors and metalloproteinases. Myeloma cells can pass back through the endothelial barrier to migrate to the peripheral blood and to mediate disease dissemination.

MRP-1 favours enhanced metastasis formation by affecting cell motility. Interestingly, we found that most MM cell lines with extramedullary origin are MRP-1 negative. These data suggest that this molecule might also play a role in the dissemination of myeloma cells and the clinical evolution of the disease.

Conclusions
Figure 3 shows a schematic overview of the different successive steps in the pathogenesis of MM. A consistent major finding is the fact that early in disease myeloma cells behave as regular end-stage B-cells homing to bone marrow and differentiating into plasma cells. Early oncogenic events will favour tumour growth by bone marrow stroma-dependent mechanisms. The functional interplay between MM cells and the host microenvironment includes paracrine interactions with stromal fibroblasts to sustain tumour survival and growth, with osteoclasts and osteoblasts to induce bone-remodelling and osteolysis and with endothelial cells to mediate neovascularisation. MM clonal cells will (re)circulate by cross-endothelial migration. Once in the bone marrow the tumour clone expands by a functional interplay with the microenvironment. This communicating network includes anchoring to stromal elements, paracrine cytokine interaction, remodelling of extracellular matrix and bone, activation of osteoclasts, inhibition of osteoblasts and induction of neovascularisation. Myeloma cells can pass back through the endothelial barrier to migrate to the peripheral blood and to mediate disease dissemination.

References
12. Vande Broek I, Vanderkerken K, De Gref C et al. Laminin-1 induced migration of multiple myeloma cell lines involves the high affinity 67KD laminin receptor.
Strategies to improve the outcome of stem cell transplantation in multiple myeloma

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Experience from around the world, including our Myeloma Center, suggests that high response rates can be achieved using high dose therapy followed by stem cell grafting; however, patients are destined to relapse and few, if any, are cured.1,4 Major obstacles to cure are the excessive toxicity noted after allografting in myeloma, contaminating tumour cells in autografts, and most importantly, the persistence of minimal residual disease (MRD) after high dose therapy followed by either allogeneic or autologous stem cell transplantation. In this context, we are developing improved strategies to treat MRD after high dose therapy followed by allogeneic or autologous stem cell grafting.6 Most importantly, we are developing multiple approaches for the generation and enhancement of allogeneic and autologous anti-myeloma immunity in vitro and in animal models. Based upon these studies, we are designing clinical trials which couple our treatments to achieve MRD with these novel immune-based therapies for MRD post-transplant in an attempt to achieve long-term disease free survival and potential cure of multiple myeloma.

Allografting

We have carried out high dose therapy followed by T (CD6) cell depleted allografting using histocompatible sibling donors in 61 patients with myeloma whose disease remained sensitive to conventional chemotherapy.4 These patients comprised 39 men and 22 women with a median age of 44 (range, 32-55) years. Most patients presented with advanced stage myeloma. The majority of patients achieved either complete (28%) or partial (57%) response; importantly, only 17% patients developed ≥ grade 2 graft-versus-host disease (GVHD), and the transplant-related mortality was only 5%. We have, therefore, shown that allografting can be done safely in myeloma. Indeed in our centre the overall and progression-free survivals of allograft and autograft recipients are equivalent, with approximately 40% patients surviving at 3 years. However, only 20% patients are disease-free 4 or more years after transplantation. Excitingly, data from our4 and other6 centres unequivocally demonstrate that donor lymphocyte infusions (DLI) mediate a graft-versus-myeloma effect (GVM) which can effectively treat relapsed myeloma post-allografting. Unfortunately GVHD is a frequent cause of morbidity and mortality after DLI. However, at our Myeloma Centre 5 of 7 patients who had relapsed post-CD6-depleted allografting responded (including 3 complete responses) to CD4+ T-cell enriched DLI, in some cases in the absence of GVHD.9 This raised the possibility that distinct T-cell clones may be mediating GVM versus GVHD. Given the high response rates but inevitable relapses observed in the setting of allografting for myeloma, we are now testing in a clinical protocol whether CD4+ DLI 6 months after CD6 depleted allografting may mediate GVM which will effectively treat MRD and thereby improve outcome. To date 21 patients have undergone CD6 depleted allografting, 18 of whom developed only grades 0-1 GVHD. Eleven of these 18 patients had their transplant more than 6 months ago and have received CD4+ DLI. Eight of these 11 patients who have already received DLI demonstrated a further response (including 4 complete responses), suggesting the potential of DLI to treat MRD. Our studies, therefore, already suggest that GVM can be adoptively transferred in this fashion. We are also examining T-cell repertoire, based upon Vβ T-cell receptor gene rearrangement, to identify those clonal T-cells associated with GVM and their target antigens on tumor cells. We have already shown that T-cells mediating GVM can target idiootypic antigens, and we are presently identifying other target antigens. The goal of these studies is to characterise, isolate, and expand GVM T-cell clones for antigen-specific adoptive immunotherapy.

Autografting

Although randomised studies convincingly demonstrate a survival advantage for myeloma patients treated with high dose therapy and autografting compared to those receiving conventional chemotherapy, this treatment is not curative.7,10 Two sites of MRD contribute to the failure of autografting: MRD in the autograft; and MRD in the patient post-myeloablative therapy. At our Center we have to date carried out high dose therapy and stem cell autografting in 105 patients who presented with advanced stage myeloma but whose disease remained sensitive to chemotherapy.6 As in our allografting experience, the majority of patients responded, including 30% complete and 62% partial responses. However, none of these patients is cured. We have produced monoclonal antibodies in the laboratory which have been used to deplete tumour cells from myeloma autografts.4 We have also evaluated CD34 selection tech-
niques to select normal haematopoietic progenitor cells within autografts.\(^{11}\) However, each of these methods depletes only 2-3 logs of tumour cells, and more than 50% of autografts still contain MRD. Based upon our laboratory data that myeloma cells express M uc-1\(^{12}\) and adenoviral receptors,\(^{21}\) we have specifically transduced tumour cells within myeloma autografts with the thymidine kinase (TK) gene using an adenoviral vector with a tumour selective (M uc-1) promoter, followed by purging tumour cells ex vivo by treatment with ganciclovir. Pilot studies suggest that >6-7 logs of tumour cells can be purged under conditions which do not adversely affect normal haematopoietic progenitor cells, setting the stage for a clinical trial of adenoviral purging prior to autotransplantation. We are also attempting to generate and expand anti-myeloma specific autologous T-cells ex vivo for adoptive immunotherapy of MRD in the patient post-autotransplant. It is now possible to clone the gene for the patient's specific idiotypic protein, use computer programmes to identify gene sequences encoding for peptides predicted to be presented within the groove of class I HLA of a given patient's HLA type, and expand peptide specific T cells ex vivo.\(^{14,15}\) A similar strategy can be used to expand T-cells against peptides within shared antigens which are overexpressed on myeloma cells, such as telomerase catalytic subunit (hTERT), M uc-1, or CYP1B1.\(^{12,16,17}\) Strategies are being tested to enhance the immunogenicity of the whole tumor cell. Our laboratory studies have shown that autologous T-cells do not proliferate to the patients' own tumour cells as targets in an autologous MLR. However, CD40 activation of myeloma cells upregulates class I and II HLA, co-stimulatory, GRP94, and other molecules, and that CD40 activated myeloma cells trigger a brisk autologous T-cell response.\(^{18,19}\) T-cells can therefore be harvested from myeloma patients before autotransplanting, expanded ex vivo using CD40 activated autologous myeloma cells as stimulus, and given as adoptive immunotherapy to treat post-transplant MRD. Finally, we are developing and examining the clinical utility of a variety of myeloma vaccines. First, based upon our observation that CD40 activated myeloma cells trigger a brisk autologous T-cell response,\(^{18,19}\) we will examine the utility of vaccinating patients with autologous CD40 activated tumour cells; we have recently developed systems for purifying patient's tumour cells for this purpose.\(^{20}\) Second, based upon our demonstration of the expression of M uc-1 core protein on freshly isolated myeloma cells,\(^{12}\) we will construct and evaluate two vaccines: recombinant vaccinia virus containing the M uc-1 gene; and autologous dendritic cells (DCs) transduced using adenoviral vectors with M uc-1. Excitingly, we have recently shown that myeloma cells can be fused to DCs and that the use of the myeloma cell-DC fusion as an antigen presenting cell presents the entire myeloma cell as foreign. In a syngeneic murine myeloma model, vaccinations with myeloma cell-DC fusions, but not with either myeloma cells or DCs alone, demonstrate both protective and therapeutic efficacy. Most importantly, we have shown that patient myeloma cells can be fused to autologous DCs, which are readily isolated from either patient's bone marrow or peripheral blood,\(^{21}\) and that autologous myeloma cell-DC fusions can trigger specific autologous T-cell responses in vitro. We will therefore translate these findings to the bedside in clinical trials of myeloma-DC vaccine fusion to assess in vivo myeloma-specific T- and B-cell responses, as well as clinical efficacy. Ultimately, vaccinations will be coupled with adoptive immunotherapy in an attempt to treat post-autografting MRD and thereby improve patients' outcome.

Manipulating the tumour microenvironment

Recent studies suggest that adhesion of myeloma cells to extracellular matrix proteins and bone marrow stromal cells confers resistance to apoptosis, suggesting therapeutic strategies of blocking adhesion and thereby restoring tumour cell sensitivity.\(^{22}\) Delineation of the cascades whereby myeloma cells escape apoptotic signals also generates novel therapies.\(^{23}\) If a pathogenic role for human herpesvirus-8 is identified in myeloma, this will represent another novel therapeutic target.\(^{24,25}\) Finally, the recent exciting data that angiogenesis may be increased in myeloma,\(^{26}\) coupled with the anti-angiogenic activity of thalidomide,\(^{27}\) provide the framework for new treatment approaches. There are multiple potential mechanisms of action of thalidomide and/or its in vivo metabolites in the setting of myeloma.\(^{28}\) First, thalidomide may have a direct effect on the myeloma cell and/or BM SC cell inhibiting their growth and survival. For example, free radical-mediated oxidative DNA damage may play a role in the teratogenicity of thalidomide,\(^{29}\) and may also have anti-tumour effects. Second, adhesion of myeloma cells to BM stromal cells both triggers secretion of cytokines which augment myeloma cell growth and survival and confers drug resistance; thalidomide modulates adhesion interactions\(^{30}\) and thereby may alter tumour cell growth, survival, and drug resistance. Third, cytokines such as interleukin-6 (IL-6), IL-18, IL-10 and tumor necrosis factor (TNF)-\(\alpha\), secreted into the BM microenvironment by myeloma and/or BMSCs, may augment myeloma cell growth and survival,\(^{31}\) and thalidomide may alter their secretion and bioactivity.\(^{32}\) Fourth, VEGF and basic fibroblast growth factor (bFGF)-2 are secreted by myeloma and/or BMSCs and may play a role both in tumor cell growth and survival, as well as BM angiogenesis. Given its known anti-angiogenic activity,\(^{33}\) thalidomide may inhibit activity of VEGF, bFGF-2, and/or angiogenesis in myeloma.\(^{34}\) Finally, thalidomide may be acting against myeloma via its immunomodulatory effects, such as induction of a Th1 T cell response with secretion of interferon-\(\gamma\) (IFN-\(\gamma\)) and IL-2.\(^{35}\) Understanding which of these mechanisms mediate anti-myeloma activity will be critical both for optimal definition of this drug's clinical utility and to derive analogues with enhanced potency and fewer side effects. Already two classes of thalidomide analogues have been reported, including phosphodiesterase 4 inhibitors which inhibit TNF\(\alpha\), but have little effect on T-cell activation, and others which are not phosphodiesterase inhibitors but do markedly stimulate T-cell proliferation as well as IFN-\(\gamma\) and IL-2 secretion.\(^{32}\) Identification of the mechanisms mediating anti-myeloma effects will not only provide insights into...
myeloma biology, but also suggest novel anti-angiogenic and/or immune-based treatments.

Conclusions
Although stem cell transplantation is currently not curative in myeloma, development of mechanisms for enhancing allo and autoreactivity to myeloma cells will provide the framework for novel adoptive immunotherapy and vaccination trials to treat post-transplant MRD. In addition, characterisation of cellular and molecular mechanisms regulating myeloma cell growth and resistance to apoptosis in vitro, including factors such as angiogenesis in the BM microenvironment, will provide the framework for novel therapies to inhibit tumor cell growth specifically and/or trigger apoptosis. These therapies will not only target the tumor cell directly, but also indirectly via inhibition of accessory factors in the BM milieu. Immune and biologically-based treatment approaches, either alone or together, offer great potential to improve the outcome of stem cell transplantation in myeloma.

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References
The role of autologous stem cell transplantation in multiple myeloma

In the absence of any significant improvement of conventional chemotherapy (CC), high dose therapy (HDT) with autologous stem cell transplantation (ASCT) has been increasingly used in the past 15 years in multiple myeloma (MM). Uncontrolled studies have shown that, for patients responding to initial induction chemotherapy, ASCT is a safe (less than 5% toxic deaths) and effective consolidation therapy. Most importantly, some of these studies have suggested that 30-50% complete remissions (CR) could be achieved with this approach in patients with newly diagnosed MM and that this more important tumor burden reduction could be converted into a prolongation of remission and of survival. However, these pilot studies are difficult to analyze because the recruitment of patients is subject to selection bias regarding age, performance status, renal function and response to initial chemotherapy. Historical comparisons have suggested that survival of patients less than 65 years of age responding to initial chemotherapy and receiving only CC was similar to that reported in selected series of patients giving early HDT. Therefore, prospective randomised trials were needed to compare CC with HDT. In 1990, the Intergroupe Français du Myélome (IFM) began a trial designed to address this issue. The results of this trial were published in 1996 but they have been recently updated. With a median follow-up of 60 months, the new analysis of the IFM 90 trial confirms the previously published results. In this study, the time of diagnosis, patients less than 65 years of age with Durie-Salmon stage II or III MM were randomly assigned to receive either CC or HDT.

The intent-to-treat analysis showed a significant superiority of HDT over CC regarding the response rate (38% CR plus very good partial remission versus 14%), the 6-year event-free survival (EFS) (25% versus 14%) and overall survival (OS) (43% versus 28%), although 26% of the patients did not undergo the planned autologous transplantation. As the CC arm of this trial was a commonly used regimen and yielded results comparable to those published in the literature, this trial demonstrates that HDT represents a significant improvement and should be proposed as part of front line therapy in younger patients.

A retrospective comparison of patients undergoing a programme of double ASCT and matched patients treated with CC according to the SWOG protocols led to similar conclusions. However, Fernand et al. recently reported the results of another randomised French trial. In this study 190 patients aged 55 to 65 years were randomised to receive CC or HDT. Although, the results of HDT appeared comparable to those achieved in the IFM 90 trial, there was no significant difference in overall survival between the 2 arms, due to an unexpected survival in the CC arm (median 55 months with HDT versus 50 months with CC). It should be noted that in the CC arm, 17 patients received ASCT at the time of relapse.

How to improve the results of ASCT?

In the IFM 90 trial, the 6-year EFS is only 25% in the HDT arm and there is no plateau of the survival curves. Therefore strategies to improve these results are clearly warranted. Since in this trial, achievement of CR is significantly associated with a prolongation of survival, the aim of ongoing studies is to increase the CR rate.

The conditioning regimen

Improving the conditioning regimen could be one way to attain this objective. The optimal conditioning regimen for ASCT in MM has not yet been determined. Since its introduction in 1987 by Barlogie et al., total body irradiation (TBI) has been used in many uncontrolled studies. The combination of TBI plus HDM 140 mg/m² yields CR rates ranging from 20 to 50% according to the disease status at transplantation and to criteria used to define CR. This conditioning regimen was used in the IFM 90 trial and could therefore be considered as the standard one. However, in newly diagnosed patients, the Royal Marsden Group has reported an impressive 70% CR rate with HDM 200 mg/m², with a low extramedullary toxicity.

Therefore, in 1995 the IFM initiated a randomized study comparing HDM 200 mg/m² and HDM 140 mg/m² plus TBI followed by peripheral blood progenitor cell (PBPC) autologous transplantation in patients with newly diagnosed MM. So far, 386 patients have been currently enrolled in this study. A preliminary analysis of the first 221 patients has been performed. Currently, there is no significant difference in the outcome between the two groups. However, HDM 200 mg/m² appears to be less toxic (shorter time to haematopoietic recovery, fewer cases of grade ≥3 mucositis, fewer toxic deaths). If these results are confirmed by further analysis, HDM 200 mg/m² should be preferred to the standard regimen HDM 140 mg/m² plus TBI.
There is a clear dose-effect relationship with melphalan but the maximum tolerated dose of intravenous melphalan is unknown. In a preliminary study, Moreau et al. have shown that a dose of 220 mg/m² increases the area under the curve of plasma concentration without modifying other pharmacokinetic parameters. 

6. Except for a high incidence of severe mucositis this regimen was well tolerated when supported by PBPC transplantation.

The impact of tandem transplants

Another way to increase the CR rate could be to repeat intensive treatments. We were the first to explore this strategy but the haematopoietic toxicity of the first course of HDT was severe in the absence of any haematopoietic support. Thanks to autologous transplantation of PBPC and to haematopoietic growth factors, the sequential use of several courses of HDT appears to be well tolerated. The Seattle group reported results achieved in 55 patients treated with two cycles of HDT 200 mg/m². They showed that tandem HDM with PBPC transplants can be administered to 70% of patients 65 years old and younger and that the CR rate increases from 15% after the first cycle to 55% after the second.

The largest experience in this setting comes from the Little Rock Group. Out of 495 patients enrolled to undergo two transplants including 315 pretreated patients, 95% completed the first course of HDM 200 mg/m² with PBPC transplantation and 75% two transplants. The CR rate increased from 24% after the first transplant to 43% after two transplants. This experience has now been extended to more than 1,000 patients.

However, the real impact of such an aggressive strategy on EFS and OS needed further evaluation. In 1994, the IFM initiated a randomised trial (IFM 94) comparing one versus two transplants. From October 1994 to March 1997, 405 untreated patients under the age of 60 years were enrolled by 36 centres. At diagnosis the patients were randomized to receive either a single ASCT prepared with melphalan (140 mg/m²) and TBI (8 Gy) or a double ASCT: the first one prepared with melphalan (140 mg/m²) and the second one prepared with melphalan (140 mg/m²) and TBI (8 Gy). Patients were initially treated with 3-4 cycles of the VAD regimen. An interim analysis was performed on May 1999 in the 402 eligible patients with a median follow-up of 36 months.

On an intent-to-treat analysis, there was no significant difference between one and two ASCT regarding the CR rate, (32% versus 35%) the 3-year EFS (31% versus 39%) and the 3-year OS (58% versus 66%). However, it should be emphasised that these results are preliminary. Since there is a trend in favour of two transplants, longer follow-up is needed before drawing any definite conclusion.

The source of stem cells

As in other malignancies, PBPC have almost completely replaced bone marrow as the source of stem cells in ASCT for MM. The main reasons for this choice are easier accessibility and availability, faster haematopoietic recovery and possibly lower tumour contamination. However, several issues remain regarding the use of PBPC. Although tumour cell contamination is lower in PBPC harvests than in bone marrow, the superiority of PBPC autologous transplantation as regards the clinical outcome has not yet been demonstrated.

A retrospective case-control study of 132 patients transplanted in 18 French centres showed that there was no difference in overall response rate, CR rate, EFS or OS. The only advantage of PBPC transplantation was a significant reduction in the median duration of neutropenia. However this study was not randomised and growth factors were not used for either PBPC collection or after ASCT.

In order to answer the question of the prognostic impact of PBPC as compared to bone marrow, patients included in the IFM 94 trial were randomly assigned to receive PBPC or bone marrow to support a conditioning regimen with HDM 140 mg/m² and TBI. PBPC were collected with granulocyte colony-stimulating factor (G-CSF) alone in steady-state after VAD therapy. 

Growth factors were administered after ASCT. A preliminary analysis of this study first shows that a large number of patients allocated to receive bone marrow have actually received PBPC because of the patient’s or the investigator’s choice. Thus 145 PBPC transplantations are compared to only 97 autologous bone marrow transplantations. The use of PBPC significantly reduces the mean duration of neutropenia, the mean duration of thrombocytopenia and the mean number of platelet transfusions. However, there is no significant difference between the 2 arms regarding the immediate outcome, the EFS and the OS.

The fact that PBPC transplantation does not prolong EFS and OS in spite of a lower tumour load in the graft could mean that relapse is mainly explained by the persistence of clonogenic malignant cells in the patients after HDT. If these results are confirmed by further analysis, the current choice in favour of PBPC will be justified only by a more rapid haematopoietic reconstitution.

Sensitive immunofluorescence studies or polymerase chain reaction (PCR) based techniques have demonstrated that virtually all PBPC harvests are contaminated by malignant cells. Although the prognostic significance of detecting malignant cells with such sensitive methods is still unknown, efforts to reduce tumour cell contamination of the grafts has been great.

Attempts to purge marrow with cyclophosphamide derivates or with monoclonal antibodies have proven feasible although they induce prolonged myelosuppression. Selection of CD34⁺ progenitors appears to be a promising alternative since positive selection of CD34⁺ cells results in 2.5 to 4.5 log-depletion of plasma-cells. Several pilot studies have confirmed the feasibility of autologous transplants with CD34⁺ selected PBPC in MM. A multicentre phase III trial comparing selected and unselected PBPC in 131 myeloma patients was recently published. Successful neutrophil engraftment was achieved in all patients by day 15 and there was no significant difference be-
randomised study has so far been completed. This in patients with minimal residual disease. Only one HDT in the hypothesis that it could be more effective remission duration by 5 to 12 months as compared to unmanipulated PBPC grafts.\(^2\)

Engraftment was substantially delayed in these grafts as compared to unmanipulated PBPC grafts.\(^3\)

**Maintenance therapy**

As there is no plateau of the survival curves in published series with adequate follow-up, some form of maintenance therapy appears necessary. Several randomised studies have shown that, in patients responding to CC, α-interferon maintenance prolongs remission duration by 5 to 12 months as compared to observation. α-interferon has also been used after HDT in the hypothesis that it could be more effective in patients with minimal residual disease. Only one randomised study has so far been completed.\(^4\) This trial compared α-interferon (3 × 10^6IU/kg, 3 times weekly) following recovery from HDT and no further therapy. With a median follow-up of 77 months, the median PFS was significantly longer (42 months versus 27 months for the control arm), but the PFS and OS curves, which were better with interferon at the time of first analysis, are no longer significantly different. This means that, although interferon delays relapse, especially in patients in CR, most if not all patients ultimately relapse. However, since this study involved only 85 patients, these results should be interpreted cautiously. Further studies are needed and a large randomized trial is ongoing in the US. It should be noticed that, in the IFM 90 trial, although α-interferon was to be administered to all patients after HDT, there is not plateau of the EFS curve. Therefore, it is unlikely that patients with MM treated with a single course of HDT followed by α-interferon are cured. New strategies to control minimal residual disease after ASCT are necessary.

**Current issues in ASCT for multiple myeloma**

**Timing of transplantation**

Since ASCT is also a useful salvage therapy for primary refractory MM and for chemosensitive relapses, the optimal timing of ASCT is not yet known. Fermand et al. recently published the results of a randomised study showing no significant difference in OS between early ASCT and late ASCT (performed as rescue treatment in cases of primary resistance or at relapse).\(^5\)

Prognostic factors in patients with de novo multiple myeloma

Prognostic factors were analysed in two large prospective studies in which patients with de novo MM were homogeneously treated.

In the preliminary analysis of the IFM 94 trial, comparing one and two ASCT, univariate analysis showed that the initial β₂-microglobulin level, the initial level of C-reactive protein (CRP) level, the response to CC prior to ASCT and the response obtained after ASCT were significantly related to OS.

However, in multivariate analysis the initial β₂-microglobulin level was the only significant prognostic factor. In the group of patients with a good prognosis (β₂-microglobulin level ≤ 3 mg/L) the probabilities of survival at 3 years appeared to be higher for patients in the double transplant arm. Conversely, for patients with a poor prognosis (β₂-microglobulin > 3 mg/L), the 3-year OS was not significantly different between the two arms.

Barlogie et al. recently published the results of total therapy with tandem transplants in 231 patients with newly diagnosed MM.\(^6\) In multivariate analysis, better EFS and OS were observed in the absence of unfavourable karyotypes (11q breakpoints and/or partial or complete deletion of chromosome 13) and with low β₂-microglobulin level at diagnosis (≤ 4 mg/L). When combining these factors, a subgroup of patients with a very poor prognosis was identified: patients with unfavourable cytogenetics and a β₂-microglobulin level > 4 mg/L had a median survival of only 2.1 years, compared to 7 years for the remaining patients. New therapeutic strategies are clearly needed for these patients.

Using a larger cohort of 1,000 consecutive patients, including previously treated patients, the same authors confirmed that independent favorable features were mainly the absence of chromosome 13 deletion, low β₂-microglobulin level, plus low CRP level and less than 12 months of prior CC.\(^7\) Plateau of the EFS and OS curves were noted in 45 and 60% of patients with all these favourable characteristics. Thus, durable remissions and possibly cures can be achieved in a high proportion of good risk patients with an intensive strategy including tandem ASCT.

In a recent retrospective analysis of 110 patients treated with HDT, the IFM showed that the detection of chromosome 13 abnormalities (-13, 13 q-) by FISH was the most powerful adverse prognostic factor.\(^8\) The combination of FISH analysis, β₂-microglobulin and IgA isotype produced a very powerful staging system in the context of HDT. Again, patients with a high β₂-microglobulin level and chromosome 13 abnormalities had a very poor prognosis.

**Selection of patients**

Usually, ASCT is limited to patients up to 65 years of age, with performance status 0-2 and with a normal renal function. The issue of age limits was highlighted by the IFM 90 trial, since ASCT was actually performed in 82% of patients 60 years of age or less, versus only 58% in patients aged 60-65.\(^9\) As a consequence, in the intention-to-treat analysis, ASCT was significantly better than CC only in younger patients. However, the introduction of hematopoietic growth factors has profoundly modified the practice of ASCT. With PBPC collected after priming with G-CSF or GM-CSF, ASCT has become safer and could be offered to older patients. Recently, the Little Rock

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5th Congress of the European Haematology Association - Educational Book
group has compared the outcome of 49 patients 65 years and older with 49 younger pair mates selected from a cohort of 550 patients treated with HDT. The CR rate was higher in younger patients (43% versus 20%, p=0.02) and the transplant-related mortality appeared to be higher in older patients (8% versus 2%). However, since the EFS and OS were comparable, the authors concluded that age is not a biologically adverse parameter for patients treated with HDT and PBPC support and should not constitute an exclusion criterion from participation in what appears to be better therapy in MM.

In a recent retrospective analysis of 952 patients enrolled in tandem transplant trials, the same group showed that age was not a significant prognostic factor. In three subgroups defined by the b2 microglobulin level and the presence of chromosome 13 abnormalities, survival was identical for patients <65 years and for patients ≥65 years. However, the selection criteria in these studies are unknown. The toxicity and clinical impact of ASCT in patients over 60 years of age is still to be determined.

Patients with renal failure are usually excluded from HDT protocols because of an expected higher rate of complications. The Little Rock group also compared the outcome of 42 patients with renal failure with that of 84 pair-matched controls receiving the same treatment (melphalan 200 mg/m²). They showed that renal function improved in half of the patients, including dialysis-dependent patients, and that the overall prognosis was comparable to that of the controls. They concluded that renal failure should not disqualify myeloma patients from receiving HDT.

References

12. Weaver CH, Zhen B, Schwartzberg LS, et al. Phase I-II evaluation of rapid sequence tandem high-dose mel-

Session 3 – Myeloma


Treatment of acute myeloid leukemia

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Steady improvement has been observed in the last twenty years in the overall survival of patients with acute myeloid leukaemia (AML) who have entered clinical trials. This is particularly true in adults under 60 years old (Figure 1) and children1-4 but considerably less obvious in patients over 60 years old5,6 (Figure 2). These data take no account of patients who do not enter clinical trials, which is the majority of older patients. Scanty but important population based data suggest that trial data are fairly representative of what actually happens in younger patients, a high proportion of whom either enter trials or follow a similar treatment schedule.7 Only a minority of older patients enter trials and presumably are those in the best clinical conditions; since good performance score is a favourable prognostic factor, they form a highly selected group.

Treatment of younger patients

It has become increasingly common to divide patients into two age groups (<60 years or >60 years) when devising clinical trials. Any age cut-off is artificial and there will be several patients each side of the threshold who would have benefitted more from the alternative schedule. The 60-year threshold derived from a perception and habit that all treatment options including stem cell transplantation were available.

Induction treatment

Several schedules have been able to deliver remission rates of 75%-80% in patients under 60 years old.5,7 The cornerstones of treatment remain daunorubicin and cytosine-arabinoside. A third drug e.g. etoposide or thioguanine is often used. The case for etoposide was boosted by the results of the Australian Leukaemia Study Group,8 who, although not demonstrating an improved remission rate or overall survival (OS), showed improved disease-free survival (DFS) when etoposide was added to a standard DA 3+7 schedule. However, other trials have consistently demonstrated an equivalent survival with a two-drug schedule. The evidence to support the addition of thioguanine is weak. One formal comparison - admittedly from several years ago - did not show benefit. Several groups, in particular the MRC, have achieved good results including it in a 10-day schedule in several thousand patients.1 Since there is a dose-response effect of Ara-C in AML the induction dose of Ara-C has been tested in the induction phase. The AMLG demonstrated that high dose AraC (HD Ara-C) improved remission rate, DFS and OS.9 Using a similar 3 g/m² schedule a SWOG study was not able to demonstrate an improved CR or longer-term outcome.10 Measuring the benefit of intensifying induction therapy is not only about remission rates. It is not surprising that inducing a better quality of remission i.e. enhancing cytoreduction, it will favourably influence DFS. Several trials, including the Ara-C dose escalation trials, have demonstrated this principle.9,10 Dose escalation of induction phase treatment may compromise the delivery of planned consolidation treatment as was demonstrated in one of these studies.10 There is considerable scope for optimising the induction dose of Ara-C by evaluating intermediate doses.

Several trials have examined alternatives to daunorubicin. Five randomised comparing daunorubicin with idarubicin in a total of 1,900 patients were included in an Oxford Meta-analysis.11 In these trials the overall remission rate (62% vs 53%) favoured idarubicin. The disease-free survival (13.7% vs 10.4%) at 5 years almost achieved statistical significance, which converted to a small but significant survival advantage (p=0.07). The benefit was confined to patients <60 years old. A similar meta-analysis of the then 3 trials comparing mitoxantrone, showed an overall benefit for the mitoxantrone, but most patients were in the older group.10,12,13 A recent MRC trial in patients <60 years old (MRC12) in 1,300 patients was not able to show any difference between daunorubicin (50 mg/m²) and mitoxantrone (12 mg/m²) in terms of CR, DFS or overall survival. There was considerably greater myelotoxicity.14 Many trials have evaluated myeloid growth factors in the supportive care setting in AML. In spite of clear evidence that neutropenia can be curtailed, there is no consistent evidence of improvement in disease outcome. Relatively few studies have been conducted to resolve whether myeloid growth factors could be useful in priming leukaemic cells and thereby increasing sensitivity to chemotherapy.

Factors influencing response to induction therapy

Several factors can influence the response to induction treatment. Age, even in the under 60 year cohort, appears to be an independent factor even when correlations are made for other factors such as cy-
An antecedent haematological disorder or leukaemia secondary to chemotherapy is uncommon in younger patients and appears to reduce all disease outcome measures by about 20%. Cytogenetics can identify a group with an unfavourable prognosis - often coincident with the secondary AMls - who have a remission rate of around 50-60%. Patients with genetic lesions involving the core binding factor leukemia's t(8;21) and inv(16) can reliably achieve CR in 95% of cases. Poor performance score based on a WHO scale is not common in younger patients, but as in the older patients, is an adverse factor. A high WBC on presentation negatively affects CR and survival. The threshold level at which this becomes an independent variable is not agreed but is in the range of 50-100x10^9/L cells. It should be noted that in acute promyelocytic leukaemia (APL) this threshold is lower. When a patient has a very high count (>100x10^9/L cells) and a low platelet count the risk of haemorrhagic death is high - particularly as a result of intracranial bleeds.

Overexpression of markers of chemoresistance (Pgp; MRP; LRP) may be less frequent and influential in younger patients. Combination of over expression may be more important.

At present only two factors are likely to change the approach to induction treatment. If patients have APL they must have retinoic acid with conventional chemotherapy or an anthracycline alone schedule. Patients with a very high WBC need special care. Leukopheresis has been used but the evidence that this is beneficial is scanty. Count reduction with hydroxyurea is common practice and may be the least risky approach.

Prevention of relapse

Now that most patients will enter remission, prevention of relapse remains the main challenge. Fifty to 60 percent of patients who receive a transplant will become long-term survivors. Of the patients who do not receive a transplant about 40% will survive. However it is now established that the recipients of transplants are a selected minority. Several careful comparisons have been undertaken in the last decade by the major collaborative groups. The net result of these efforts is that both allo- and auto- transplantation reduce the risk of relapse. However, because they have associated morbidity and mortality which is greater than chemotherapy alone, and because these studies have demonstrated that patients who relapse from chemotherapy can be salvaged - usually by transplant - in second remission, the overall impact on survival is difficult to detect. A feature of the prospective trial experience is that the ability to deliver transplantation was relatively low, thus potentially limiting this approach. However it may be that allo-transplantation could be more effective if delivered earlier in the treatment plan. Preliminary experience suggests PBSC as a source of stem cells may improve survival, and this may be an issue that merits prospective trial comparison. Modification to the autograft approach may also be worthwhile. An obvious issue is whether PBSC is superior to bone marrow as a stem cell source. This is based on the experience that much of the 10-12% non-leukaemic mortality after autograft is associated with poor haemopoietic recovery. It is clear in AML that neutrophil recovery is more rapid but data so far are less conclusive for platelet regeneration. It is conceivable that patients whose stem cells mobilise well are at a different risk from those whose cells do not. Whether or not survival is improved by the PBSC approach is a key issue which has been evaluated in a recently concluded EORTC trial, now in the follow-up phase.

Good risk disease

The risk of relapse is highly variable and groups can be defined which range from having a poor prognosis (<20% 5-year survival) to a good risk (70% 5 year survival). The most powerful discriminant of these groups is cytogenetics. There is general agreement, drawn from the collaborative groups’ experience, that t(8;21) and inv(16) are the favourable groups and that APL t(15;17) when treated with ATRA and chemotherapy is highly favourable. The largest dataset available demonstrates that additional associated abnormalities do not detract from
the favourable prognosis. Almost certainly there are subgroups of patients within this favourable category who respond less well. Inevitably these patients are uncommon which means that it is difficult to be sure that an adverse influence is true. This has been demonstrated in the case of additional cytogenetics e.g. del. 9q in t(8;21) disease which was reported to be adverse in small series but not in the largest series. High white cell count (>50 x 10^9/L), especially with thrombocytopenia, may be adverse in APL; CD56 expression in t(8;21) are among the other reported factors. Higher age has a powerful adverse effect in AML overall but is less influential in favourable disease.

The good risk definition is not treatment independent. Clearly ATRA has been a major influence on APL. While additional chemotherapy is needed, it may be possible to reduce its toxicity substantially by removing Ara-C from the schedule. High dose Ara-C has been suggested to benefit t(8;21) disease particularly, but since alternative approaches not containing high dose Ara-C achieve comparable results, the effect may be one of treatment intensity.

Together good risk disease constitutes about 25% of AML in the <60 year old age range. Some patients with good risk are not identified because cytogenetic studies fail - usually for technical reasons. Molecular detection can pick up more cases, and, although the number of such cases is small, they seem to respond in the same favourable way.

Molecular detection of residual disease
Since all the relevant breakpoints have been cloned, favourable disease is amenable to molecular monitoring. Some general conclusions can be drawn from this. In APL using a reverse transcriptase-polymerase chain reaction (RT-PCR) with a sensitivity of 1 in 10^4 in remission can be clinically useful. If patients, having been RT-PCR negative, become positive again, the risk of relapse is high. If the RT-PCR remains positive after consolidation the risk of subsequent relapse is high - but not certain. Unfortunately most patients who do relapse were RT-PCR negative after consolidation. RT-PCR methods with greater sensitivity e.g. 1 in 10^6, can detect more positive patients, but have a poorer predictive power. Efforts to quantify residual disease accurately by real-time PCR have not so far been shown to have superior productive value. Although there is much less evidence available for t(8;21) disease in the context of modern therapy the pattern seems similar to that for the t(15;17) experience. This technology is likely to be important in the overall management of favourable disease, but whether real-time PCR will be more useful than an appropriately timed RT-PCR remains to be clarified.

Does transplantation have a role in favourable disease?

Given that tailored treatment can result in survival of >70% of these patients it is difficult to see that transplantation is needed as part of first line management. The MRC experience has been, in an intention-to-treat analysis, that only 60% of patients received the transplant, that there was a reduced risk of relapse. However since many of these patients were young and issues such as preservation of fertility were important, it was reassuring to note that at least equivalent survival was achieved by a combination of up front chemotherapy and salvage on relapse. At least three trial groups have analysed prospective trials by cytogenetic risk group (Table 1). The MRC and EORTC-GIMEMA trials showed no overall survival benefit on donor vs no donor analysis. The US Intergroup analysis did however show benefit for transplantation over a high dose Ara-C approach. It should be remembered that the numbers of patients in these analyses are not large and this is particularly true for the US Intergroup study. This increases the risk of chance findings, which may have happened because the survival of these good risk cases receiving chemotherapy was inferior to what it should have been by definition and also to patients in the standard risk category (Table 2). The message of this experience may be that more treatment is better. It may, therefore, be possible to improve results further with more anti-leukaemic treatment, which might have less morbidity and mortality than transplantation. On the other hand PBSC allo and autografts may be much more effective than stem cells from bone marrow.

Poor risk disease
Poor risk cytogenetics always includes three abnormalities of chromosome 5, complex changes, monosomy 7 and 3q-. Some groups also include abnormalities at 11q23 although the t(9;11) may be more favourable than t(10;11). Patients with poor risk cytogenetics or those who are refractory to the first course of treatment have around a 25% less chance of entering complete remission. Even if they do, they will relapse rapidly, giv-

<table>
<thead>
<tr>
<th>Table 1. Transplant vs chemotherapy in prospective trials: good risk.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Geolam</strong></td>
</tr>
<tr>
<td>Number</td>
</tr>
<tr>
<td>Relapse %*</td>
</tr>
<tr>
<td>DFS %*</td>
</tr>
<tr>
<td>Survival %*</td>
</tr>
</tbody>
</table>

* at 5 years; p = 0.01; p = 0.04; p = 0.02.

5th Congress of the European Haematology Association - Educational Book
ing them a poor prospect of survival. The priority must be to identify such patients early and offer novel therapeutic approaches. There are few leads as to what might be effective. These patients are by definition chemoresistant and would form one of the appropriate groups in which to study resistance modulators (see below). In two of the three prospective trials analysed by risk neither allo or autograft improved survival, but in the Intergroup study allo graft was beneficial. The numbers in these analyses were very small (Table 3) and firm conclusions cannot be drawn. It is probably necessary to identify these patients early and proceed to allogeneic (including unrelated) transplant as soon as possible. However it should not be assumed that the survival would be high because of the high risk of relapse. Additional strategies such as pre-emptive donor lymphocyte infusions require investigation.

Standard risk disease

About 60% of patients under 60 years old do not fit into the good or poor risk groups and are regarded as having a standard risk. This represents a spectrum of risks with an average long-term survival of 40%. There are probably further subgroups available but no one has accumulated sufficient numbers for confident further subdivision. It is now much less clear what treatment to give these patients. As shown in Table 2 transplantation reduces the risk of relapse but does not substantially improve survival, although this was the subgroup which showed a significant advantage in the MRC trial. The potential of using PBSC as the source of stem cell rescue is not yet known in AML, but if beneficial could add weight to the evidence supporting the use of transplantation, – either allo graft or autograft, - in this group of patients. A major problem with transplant has been that only a minority of patients who could benefit from it actually receives it. The statistically proper analysis of intent to treat or donor versus no donor, may therefore disguise a more powerful benefit. Part of this problem – particularly in the MRC trial – was the protocol design, which committed patients to consolidation treatment before transplant. In the case of allo graft the value of this period of consolidation chemotherapy is not known. In the case of autograft the rationale was to provide in vivo purging.

The strategy of delaying transplant for second remission in standard risk patients is not likely to be successful because the rate of second remission is lower (Table 4). Similarly, planning transplant as the treatment of the relapse presents major logistical problems in many health care systems. It appears that the benefit of allogeneic transplant may be limited to patients up to 35 years. Optimising transplantation may therefore depend on the use of PBSC and/or delivering it early in the treatment plan.

The intensive chemotherapy approaches used in comparative trials should form the basis of further efforts to improve treatment. Many questions about HD Ara-C dose and scheduling need to be resolved. The transplant experience suggested that more treatment was better, but the issue of how many courses has not been resolved although this is a major question currently being addressed in the MRC-AML12 Trial.

AML in the older patient

The cytogenetic and risk group profile of older patients (>60 years) who enter trials is inherently more adverse than that in the younger group. The proportion of poor risk cytogenetics is higher and favourable cytogenetics lower. More patients have secondary AML. The presentation performance score is poorer, and patients may have co-morbidity, leading to poorer tolerance of the consequences of chemo-

**Table 2. Transplant vs chemotherapy in prospective trials: standard risk.**

<table>
<thead>
<tr>
<th></th>
<th>Geolam²²</th>
<th>MRC²²,²₅</th>
<th>EORTC²</th>
<th>US Intergroup²⁹</th>
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<tr>
<td>Number</td>
<td>33/35</td>
<td>97/112</td>
<td>NA</td>
<td>37/44</td>
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<tr>
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<td>194/428</td>
<td>NA</td>
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<td>DFS %*</td>
<td>39/31</td>
<td>34/56²</td>
<td>53/39¹</td>
<td>47/66</td>
</tr>
<tr>
<td>Survival %*</td>
<td>43/51</td>
<td>57/45¹</td>
<td>42/29</td>
<td>36/55</td>
</tr>
</tbody>
</table>

*at 5 years; ¹p = 0.02; ²p = 0.05; ³p = 0.003; ⁴p = 0.00002.

**Table 3. Transplant vs chemotherapy in prospective trials: poor risk.**

<table>
<thead>
<tr>
<th></th>
<th>Geolam²²</th>
<th>MRC²²,²₅</th>
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<tr>
<td>Survival %*</td>
<td>29/31</td>
<td>57/87¹</td>
<td>28/22</td>
<td>13/15</td>
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</table>

*at 5 years; ¹donor vs no donor p = 0.03.
therapy. In addition to this problem the majority of patients do not enter clinical trials presumably in part because they are thought not to be able to withstand the intensive treatment required. This means there are limitations to the extent to which data derived from trials in the elderly can be extrapolated to all patients.

If patients are considered candidates for conventional chemotherapy it is better to take a more intensive approach. However it is unlikely that more than one or two consolidation courses can be delivered which are of induction level intensity. About 50-60% of patients achieve remission but this obviously depends on patient selection. While there has been a trend for improved remission in older patients, this has not translated into an improved 5-year survival (Figure 2).

Substitution of daunorubicin in induction by either idarubicin or mitoxantrone has not improved response in older patients in spite of the fact that idarubicin may not be subject to the P-glycoprotein efflux pump mechanism. It has yet to be demonstrated that more than three courses of treatment are beneficial and a recently completed MRC Trial did not demonstrate any benefit from interferon in the maintenance phase. Maintenance therapy has become unfashionable because it has not been thought to add to intensive induction and consolidation. The recently reported EORTC-HOVON 9 Trial demonstrated a benefit, albeit small, from low dose-Ara-C maintenance. This approach may be worthy of re-evaluation in the future.

There is renewed interest in anthracycline dose in the elderly and more recently also in younger patients. An old study comparing 30 mg/m² daunorubicin with adriamycin did not demonstrate an overall survival benefit. However improvements in supportive care in the intervening period have perhaps reduced the validity of this observation. A recent study from the German AML Group demonstrated a superior remission rate using 60 mg versus 30 mg of daunorubicin.

The persistently poor outcome in the elderly and the poor recruitment of this age group to clinical trials raises a number of issues. First, how can treatment be improved if it is not possible to intensify treatment? Second, which patients benefit from currently available intensive treatment which could then be improved? Third, can better palliative approaches be developed?

### Chemoresistance

Intrinsic or induced resistance to chemotherapeutic agents can now be estimated by measurement of resistance proteins in leukaemic cells. Phenotypic and functional measurement of P-glycoprotein (Pgp) has been shown in several studies to correlate with remission achievement and in some series relapse-free and overall survival. Since Pgp is part of the protective phenotype of normal stem cells it is associated with CD34 expression and tends to be more frequent in patients with poor risk cytogenetics. There are studies that have shown it to be an independent risk factor. This protein acts as an energy dependent efflux pump which can expel drugs such as daunorubicin and etoposide. It is of particular interest in AML because of its frequency of expression (70% in elderly patients), its relationship to prognosis, and the possibility of pharmacologically blocking its action.

<table>
<thead>
<tr>
<th>Risk group</th>
<th>Good</th>
<th>Standard</th>
<th>Poor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age group</td>
<td>0-14</td>
<td>15-34</td>
<td>&gt;34</td>
</tr>
<tr>
<td>Second CR (%)</td>
<td>90</td>
<td>54</td>
<td>45</td>
</tr>
<tr>
<td>Survival from relapse at 3 yr. (%)</td>
<td>38</td>
<td>9</td>
<td>15</td>
</tr>
</tbody>
</table>

Data derived from MRC AML10.

Chemoresistance modulation

Pgp function can be blocked by a number of agents including cyclosporin and its analogue PSC-833 that has the potential advantage of being non-myelosuppressive or nephrotoxic. Plasma levels required to modulate Pgp function are achievable. Two major randomised trials have attempted to evaluate the addition of cyclosporin to a conventional daunorubicin containing schedule in poor risk (relapsed) patients with AML. The MRC AML L-R Trial used up to 10 mg/m² cyclosporin but was unable to show any benefit with respect to CR, DFS or survival. Indeed in patients over 60 years old the study was stopped early because of excess mortality in the cyclosporin arm, the causes of which were not clear. A much more encouraging outcome was seen in a SWOG study. In relapsed patients there was no significant difference in remission rate but a significant and durable advantage in DFS and survival in the cyclosporin arm. Of possible importance in this study was that the cyclosporin dose was high 17 mg/m². Cyclosporin and its analogue modify the pharmacokinetics of the target chemotherapeutic agent by slowing its excretion and increasing the area under the curve. In order to
avoid toxicity, most studies have reduced the dose of the drug – thus potentially complicating interpretation of results. In the SWOG study the daunorubicin dose was not reduced but given as a continuous infusion, thus avoiding drug peaks, which may mediate the toxicity. PSC–833 has a more favourable toxicity profile and therapeutic plasma levels are readily achievable. Preliminary studies of its use which have been reported so far in very poor risk patients do not suggest efficacy in that setting.52 Another SWOG study was closed prematurely due to excess toxicity.53 The proof of principle in the SWOG study provides justification for pursuing this approach. All-trans-retinoic acid (ATRA) has been used together with standard chemotherapy in a number of small studies. In the only randomised trial in poor prognosis cases, short-term benefits were reported but final analysis could not confirm durable benefit.54 The addition of retinoic acid to conventional therapy is being extensively investigated in current MRC trials. Modulation of LRP function in vivo is not yet possible.

Antibody directed treatment

Of imminent interest is the future availability of antibodies directed chemotherapy. An anti-CD33 antibody to which calicheamicin has been linked has been developed by Wyeth-Ayerst.55 Anti-CD33 is expressed on 90% of AML and not on non-haemopoietic tissues. The antigen-antibody complex is rapidly internalised, making it an ideal vehicle for drug. Calicheamicin intercalates into DNA once the link to the antibody has been broken. Initial clinical studies of this complex (CM-A-676) in relapsed patients have shown encouraging results in that 30% achieved a complete remission with a favourable toxicity profile. This drug may therefore represent a useful non-toxic agent e.g. for treatment of older patients, or for adding more treatment to established chemotherapy regimes in younger patients. It has so far not been used in combination with chemotherapy, so the possibility of delayed haemopoietic recovery needs to be evaluated. Leukaemic stem cells are CD33 negative, so the effect of this approach may be one of a debulking agent. Finally it may be that despite the sophisticated delivery of drug to the cell, calicheamicin may still be subject to the resistance mechanisms outlined above.

Future directions

Future improvement, as in the past, will be gradual and facilitated by widespread participation in clinical trials. For younger patients the evidence supports the philosophy of more is better, but the limits of intensive chemotherapy are currently being reached. The questions on the dose and frequency of Ara-C induction and consolidation need to be resolved. A risk-directed approach to treatment will be useful if only to identify poor risk patients who require novel therapy and good risk patients who could avoid transplant. The impact of PBSC on both allogeneic and autologous transplantation has yet to be clarified, but might alter the basis on which transplant is selectively used. The concept of more treatment being deliverable without increasing toxicity by an antibody linked approach requires evaluation. Unrelated transplan-

tation will probably find a role in high risk disease and the high risk of relapse may be modified by preemptive donor lymphocyte infusion.

Treatment of older patients remains a major challenge. More attention needs to be paid to capturing information on patients who do not enter trials, and to the development of a palliative approach which can deliver a satisfactory quality of life. Modulation of chemoresistance will continue to be a potential approach, but clarification will be complicated. In particular, understanding mechanisms of resistance and circumventing not just one, but potentially combined mechanisms, will take time. The lack of significant progress in improving survival in older patients in the last quarter of a century means that we must identify patients who could benefit from an intensive or low-intensity treatment approach. At present it is possible to identify a small group of less poor risk patients by cytogenetics. Maintenance therapy in older patients may be worth re-evaluation, where a brief but intensive induction schedules has been delivered.

References


Haematopoietic stem cell transplantation with reduced conditioning: a new treatment modality for patients at high risk of transplant-related mortality

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tologeneic haematopoietic stem cell transplantation (HSCT) is a successful treatment and often the only curative approach for patients with malignant and non-malignant haematological and non-haematological diseases. Two mechanisms are thought to contribute to the success of allogeneic HSCT: the first involves cytoreductive chemoradiotherapy which is aimed at destroying tumor cells and recipient immune cells to allow engraftment; the second consists of immunological effects mediated by immunocompetent donor T-cells, referred to as graft-versus-leukemia (GvL) effects, destroys residual tumour cells. Indirect evidence for GvL was already detected decades ago by analysing experimental and clinical observations. More recently, more direct evidence has been obtained from the successful infusion of donor lymphocytes for the treatment of leukaemic relapse after allogeneic HSCT. Although the GvL effect has been recognised, it appears to vary depending on the disease under study. Best results with donor lymphocyte infusions have been seen in patients with chronic myeloid leukaemia, least effectiveness has been reported in patients with acute lymphoblastic leukaemia. The combination of chemoradiotherapy and immunological antitumour effects has been shown to result in a high percentage of disease-free survival in younger patients with HLA-matched stem cell donors. However, in older patients and in patients with concomitant adverse medical conditions, treatment-related mortality (TRM) is the factor limiting allogeneic HSCT. Even in patients with HLA matched donors, TRM may range from 20 to 73% depending on age, stage of the disease, sex, and time from diagnosis to transplant. The TRM might be even higher in patients with concomitant diseases or ages ≥50 years and, therefore, only few allogeneic HSCT have been performed in patients over 50 years old and almost none in patients over 60. Since no alternative curative approach is available for most of these patients, more specific and less toxic transplant modalities are needed to treat older patients. The relevance of the problem is easily appreciated if one considers that median ages of patients diagnosed with, say, multiple myeloma, acute myeloid leukaemia, chronic myeloid and lymphoblastic leukaemia, and non-Hodgkin’s lymphoma are between 65 and 70 years.

Given the toxicities and age restrictions of conventional transplant programmes and the clear recognition of GvL effects, attention has shifted recently towards less toxic therapies. Two different approaches have been pursued: one aims to reduce TRM by using reduced conventional conditioning regimens relying both on cytoreduction and GvL and the second more innovative approach uses minimal conditioning and relies solely on the GvL effect.

The second approach is based on extensive studies in a canine model of HSCT. Specifically, novel postgrafting immunosuppression consisting of a combination of cyclosporine (CyA) and mycophenolate mofetil (MMF) was shown not only to control graft-versus-host disease (GvHD) but also to reduce host-versus-graft (HvG) reactions. This way, it was possible to reduce the intensity of the preparative regimen needed for stable allogeneic engraftment from a single dose of 920 cGy total body irradiation (TBI) to a sublethal dose of 200 cGy.

The canine studies led to the development of new low-intensity treatment regimens suitable for older patients or those at high risk of TRM for medical reasons. The regimen consisted of 200 cGy TBI given before and CyA at 12.5 mg/kg/day p.o. from day –1 to day 35, and MMF at 15 mg/kg/day p.o. bid from day 0 to day 27 after transplant. G-CSF mobilised peripheral blood stem cells from HLA-matched donors were infused on day 0. In patients with little or no chemotherapy before transplant (e.g. CML) and those with unrelated donors (n=13) three doses of fludarabine were given before transplantation to ensure engraftment. More than 110 patients have been treated at the Fred Hutchinson Cancer Research Center in Seattle, Stanford University and at the University of Leipzig, Germany.

Among the 30 patients transplanted in Leipzig up to September 27th, 1999, 13 had AM L, 10 had CML, 3 had multiple myeloma, 2 had CLL, 1 had ALL and 1 had MDS. Thirteen patients (43%) were not in remission or chronic phase before transplantation. Their median age was 52.5 years, the range being 36 to 71 years. Seventeen received a transplant from a
sibling and thirteen from unrelated donors. Two of the unrelated donors were HLA class I antigen mismatched, the remaining were HLA class I matched by serology and HLA class II matched by DNA typing. Patients tolerated the treatment extremely well, did not experience mucositis or alopecia, had minimal nausea and vomiting, and very mild myelosuppression. Patients with near normal peripheral blood cell counts at transplantation, had no need for platelet or red blood cell transfusions.

Primary engraftment occurred in all patients. Three patients (10%) rejected their grafts and had an autologous recovery. These included one patient with AML and a history of 6 pregnancies, one patient with CML without previous cytostatic treatment and one patient given an unrelated graft with a CD34+ cell count of only 1.7 x 10^6/kg body weight. Chimerism analyses by FISH or VNTR a median of 138 days (range 41-428 days) after HSCT among all nucleated peripheral blood cells, granulocytes, T-cells and NK cells showed median values (range) of 100% (0-100), 100% (10-100) and 100% (0-100) donor cells, respectively.

Acute GvHD grade II-IV developed in 15 patients (50%) of whom 9 had grade II (30%), 4 grade III (13%) and 2 grade IV (7%) disease. Most patients developed GvHD after immunosuppression had been discontinued, and they responded to resumption of immunosuppressive treatment. Two patients with grade IV GvHD died. Donor lymphocyte infusions were given to 6 patients (20%). With a median observation time of 230 days (range 144-578 days) 5 of the 30 patients have died from relapse (16%). Five other patients died of TRM including the two patients with grade IV GvHD, one patient with sudden death (156 days after transplant), one with pneumonia and one with thrombocytopenic purpura.

Complete remissions have been observed in each of the diseases and include molecular remissions in CML (6 of 8 patients) and morphological remissions in patients with AML (12 of 12 patients), CLL (2 of 2 patients), MDS (the only patient), MM (1 of 3 patients) and in one patient with ALL. Relapses were observed in 5 patients with AML and in 2 patients with CML. One response occurred with discontinuation of immunosuppression and four after DLI.

The early results are promising and show a low rejection rate, little toxicity, a GvHD rate of approximately 50% and a remission rate of 63% in patients otherwise not eligible for conventional HSCT and in advanced stage of their disease. Disease-specific phase II studies have been initiated to evaluate the efficacy of this approach.

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References

**Therapeutic approaches to the older patient with acute myeloid leukaemia**

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**Introduction**

The majority of patients with acute myeloid leukaemia (AML) are 60 yrs of age or more. Although intensified cytotoxic therapy and bone marrow transplantation have improved the prognosis of AML in recent years in young and middle-aged adults, these therapy approaches have proved of only little benefit to individuals with AML over 60 years old, whose prognosis, therefore, is worse. The reasons for the different treatment outcome between young and middle-aged adults and older patients are probably multiple. Elderly people may suffer from AML which is a priori more resistant to chemotherapy. Furthermore, the ability of aged individuals to withstand the toxicity and morbidity caused by intensive chemotherapy is evidently far more limited.

**Remission induction treatment**

Remission induction chemotherapy usually involves a combination of the chemotherapeutic agents cytarabine and an anthracycline (daunomycin, idarubicin) or an anthrancenedione (mitoxantrone). The prevailing opinion today is that intensive chemotherapy is the treatment of choice for patients of 60 yrs and over suffering from AML, if they are fit. Following one or two cycles of chemotherapy approximately 40-55% of them may enter complete remission. Thus, it is evident that the elderly population of patients respond significantly less favourably to induction treatment. The inverse relationship between age and complete remission rate is apparent from a number of studies that enrolled patients covering a broad range of ages. These results are indicative of a progressive decline of the response to chemotherapy with increasing age. How can these differences be explained? Are they due to differences of the dose or the choice of the chemotherapeutic agents? Or are they perhaps due to the occurrence of AML of greater risk in patients aged 60 yrs and over? Only a few prospective randomised studies have addressed the question of choice of drug and choice of dose and revealed differences in efficacy. Two studies addressed the question of the possible efficacy of mitoxantrone as compared to daunomycin, but did not provide an unequivocal answer. In one study, mitoxantrone produced significantly better response rates in elderly patients. In contrast, in another study mitoxantrone and cytarabine as first-line therapy did not improve the likelihood of response. One recent phase III study involving more than 500 patients showed a slight advantage of mitoxantrone-cytarabine therapy (as compared to daunomycin-cytarabine treatment) with respect to response, but no difference with respect relapse or overall survival. A critical evaluation of the comparative value of idarubicin in older subjects has yet to be made.

**Complications and mortality during remission induction therapy**

Approximately 30% of patients who do not enter remission, fail due to early death or death during the hypoplastic phase post-chemotherapy. Death occurs during hospitalisation in spite of supportive care including antibiotics and red blood cell and platelet transfusions. The major cause of death among these individuals is infection. The greater death rate would indicate that elderly individuals are less able to tolerate the consequences of severe infections. They succumb more frequently to the negative effects on cardiac, pulmonary or renal function or haemodynamic dysfunction. The inability of elderly subjects to regenerate haemopoiesis in response to cytotoxic therapy or other perturbations, the reduced ability to regenerate marrow, the reduced tolerance of other organs is handling the toxic effects of drugs as well as the reduced elimination and metabolism of drugs might all explain why elderly patients are at increased risk of haematopoietic and other complications of intensive chemotherapy. The incidence of severe infections in elderly patients with AML on induction therapy is approximately 20%. If one considers the entire induction phase including the interval of post-chemotherapy hypoplasia approximately 20% of patients die. Apparently, a substantial proportion of over-60 years olds with AML do not survive remission induction therapy because of infectious or haemorhagic complications during or following chemotherapy. Those who experience significant toxicity following the first cycle of chemotherapy are generally withdrawn from additional efforts at treatment. The obstacles to offering adequate therapy to older patients with AML are still, apparently, quite formidable. There is hope that haematopoietic growth factors may have a role in hastening haematopoietic recovery, reducing the incidence and severity of complications and ultimately reducing mortality.

**Use of haematopoietic growth factors**

The use of haematopoietic growth factors to accelerate haematopoietic recovery and prevent morbidity has attracted wide attention. Granulocyte...
colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) can stimulate the production of granulocytes (both G-CSF and GM-CSF) and monocytes (GM-CSF), promote their mobilisation from the marrow into blood circulation and activate granulocyte and monocyte functions. Thrombopoietin and several other newly identified cytokines have more recently become available for clinical investigation in patients with AML. A substantial number of randomised studies have been completed evaluating G-CSF or GM-CSF as adjuncts to induction or consolidation cycles of chemotherapy. The duration of neutropenia was consistently shorter with use of either cytokine in these studies. This benefit translated into fewer days of antibiotic or antifungal therapy or fewer days in the hospital. None of the studies showed a reduction in the number of documented infections. In one study survival appeared to be improved. In light of the above findings, G-CSF or GM-CSF does not have a standard role in the clinical management of AML patients. However, therapeutic use of these cytokines might be justified in patients with serious infections unresponsive to antimicrobial treatment. A future role for myeloid colony-stimulating factors is suggested by studies in which peripheral blood progenitor cell autografts (to replace marrow transplants) showed accelerated haematopoietic regeneration following mobilisation with colony-stimulating factors and cypheresis.

Therapy failure
Resistance to chemotherapy has been the major cause of treatment failure in over 60-years old patients with AML. In a number of recent studies the proportion of patients showing primary resistance to chemotherapy as the cause of remission induction failure was estimated at 20% or more, and among the majority of those achieving complete remission, the leukaemia recurred within 2 yrs. Why should AML in elderly individuals be relatively unresponsive to chemotherapy? Are the intrinsic unfavourable prognostic features of AML more frequently expressed in elderly patients? Unfavourable cytogenetic abnormalities (e.g. chromosome 5 and 7 abnormalities) and MDR-1 expression are highly adverse predictors of treatment outcome. Current evidence indicates that the incidence of these drug-resistance phenotypes is higher among elderly patients with AML. The results of some retrospective studies also suggest that elderly patients generally present more often with secondary AML following myeloproliferative disorders and myelodysplasia (13% versus 4%). These subsets of patients with multidrug resistance (MDR) show reduced responses to induction chemotherapy. Favourable cytogenetic subtypes of AML (e.g. AM L M 3) are comparatively underrepresented (1% versus 12%) among patients aged 60 yrs and over. This would a priori create an unfavourable stage for treatment outcome. AMLs evolving from myelodysplasia as well as AMLs secondary to chemotherapy applied previously for another medical condition are generally poor risk conditions. The myelodysplastic syndromes are more often seen in elderly patients and are likely to contribute to the increased incidence of high risk AML in older subjects. AMLs developing after progression from MDS generally respond poorly to chemotherapy, thus providing another explanation for the unsatisfactory response to treatment.

Drug resistance
By definition, patients who relapse or fail to respond to current chemotherapy programmes show clinical resistance. There is much interest in the mechanisms of drug resistance and the extent to which they play a role in AML. Classical MDR or MDR1 is associated with the expression of the membrane marker P-glycoprotein (PgP). Because this molecule acts as a drug efflux transporter in the plasma membrane for a variety of antileukaemic drugs (e.g. anthracyclines, etoposide), high levels of expression of MDR1 have been associated with reduced intracellular concentrations of chemotherapeutic agents in tumour cells. Other genes involved in the mechanisms of resistance to chemotherapy and serving as predictors of treatment response are MRP (MDR associated protein, a transporter of the glutathione complex) and the lung resistance protein (LRP). High levels of MDR1 and reinduced drug retention are commonly seen in older patients with AML, thus explaining cross drug resistance to structurally unrelated but MDR-dependent cytotoxic agents. Older patients with AML expressing MDR1 show significantly reduced response rates to chemotherapy. Although the molecular pathways leading to the development of drug resistance in AML remain largely unknown, drugs reversing resistance are currently being developed. Early studies with inhibitors of P-glycoprotein function (e.g. cyclosporin-A and its analogues) have however yet to fulfill their therapeutic promise.

Post-induction therapy
As most older patients successfully induced into complete remission, usually relapse within one year, therapeutic efforts to maintain remission are appropriate. Commonly, following attainment of complete remission, one or two additional cycles of chemotherapy are applied, at least if no significant intercurrent medical problems have complicated the induction treatment phase. Whether moderate dose chemotherapy programmes for extended periods (e.g. up to 12 months) would be useful in older patients with AML, has remained a relevant but unresolved question. In two large studies patients in complete remission were randomised to receive low-dose cytarabine (10 mg/m² for 10 days at 6-week intervals) in an outpatient setting, there was a trend in favour of this maintenance chemotherapy. However, the results were not conclusive since the results were not statistically significantly different and only a minority of eligible patients were randomised for maintenance therapy. There is limited evidence indicating that complete responders following induction therapy, may benefit from successive cycles of comparatively low doses of chemotherapy. The current hypothesis is that such regimens add only minimal cytoreduction when applied following chemotherapy induction regimens based on 200 mg/m² cytarabine and 45 mg daunomycin or equivalent dosages.
Stem cell transplants

Bone marrow transplants are infrequently done in patients aged 60 years and above.

A randomised study of post-remission therapy with three dose levels of cytarabine (100 mg/m², 400 mg/m² and 3 g/m²) showed a clear dose-effect relationship with regards to (disease-free) survival in adults under 60 years old, but this relationship was not apparent in older subjects. The schedule of cytarabine at 3 g/m² resulted in a reduced relapse probability and longer survival in patients under 60-years old but, again, the advantage of dose escalation of cytarabine was not apparent in older individuals. Therefore, high dose chemotherapy does not appear beneficial to the elderly with AML. Since patients over 60-years olds do not tolerate intensive chemotherapy, there has been an increasing interest in the development of allogeneic transplants following non-myeloablative preparative regimens. The goal of these approaches is to establish allogeneic chimerism following immunosuppressive therapy and then exploit the graft-versus-leukaemia effects of the allografts and use donor chimerism as a platform for subsequent infusions of donor lymphocytes (DLI). Early clinical trials, for the time being based on small numbers of patients with limited follow-up, confirm the feasibility of the approach as a proof of principle. In older patients with various haematological malignancies, in (mixed) donor chimerism can indeed be established, but more long-term data are needed for a critical assessment of the clinical value of this approach.

Outcome and prognosis

Survival at 2 years in patients of 60 yrs and older treated with chemotherapy has been estimated to be approximately 20% and that at 4 to 5 yrs at 10% or less. Thus, generally only a small fraction of elderly patients submitted to intensive chemotherapy have the prospect of becoming long-term survivors. These results, while being highly unsatisfactory, probably represent an overestimate of the real disease outcome in elderly individuals with AML. Many patients are not referred to hospital for chemotherapy, others reject or refuse to be treated, or are considered ineligible to receive chemotherapy for medical reasons. If these patients are taken into account, the overall prognosis of elderly patients with AML is even more dismal than the results of prospective studies indicate. One report estimated that only approximately one third of elderly patients were selected to enter treatment protocols. In fact, the variability of patient selection leading to the inclusion of patients with different risks into individual studies might help to explain the differing outcomes of treatment in study reports. The decision to treat or not to treat is important in the elderly individuals. It makes sense to select patients for induction treatment only if they are in reasonably good clinical condition. Further more one could argue that it makes sense to proceed with additional cycles of therapy only if they tolerate the first cycle of treatment with no major complications and if a haematological response to the first therapy cycle is evident. This practical approach may serve to avoid overtreatment in elderly patients, i.e. treatment with no benefit at the expense of prolonged hospitalisation.

Summary

Sixty-year olds living in western countries generally have a mean life expectancy of at least 20 yrs. Therefore, when elderly individuals present with AML, it is a necessity and a challenge to treat them as effectively as possible. AML is mainly a disease of the elderly who account for more than 50% of its incidence among the general population. The treatment of older individuals with AML remains difficult and of still limited success. While complete responses above 65% and survival rates of 35% are commonly obtained in adults with AML under 60 years old, progress in the treatment of elderly patients has been relatively small. Approximately 50% of older patients may be induced into remission with chemotherapy but of these complete responders only approximately 1 in 10 will survive free of leukaemia beyond 4 yrs after diagnosis. These results represent the rationale and motivation for offering chemotherapy to the older population, but they also emphasise that major obstacles to better cure rates still exist. These stumbling blocks are apparently related to the reduced tolerance of older subjects to chemotherapy and probably also a greater probability of unresponsiveness of the leukaemia to cytotoxic therapy. The use of haematopoietic growth factors still holds some promise and may improve outcome, but for the time being there is insufficient direct evidence to indicate a defined and established role. It is evident that new avenues should be followed and trials specifically designed for elderly people with AML be conducted. These trials need to address questions related to the choice of chemotherapeutic drugs (e.g. idarubicin versus mitoxantrone), their dose and schedule, the use of multidrug resistance modulators (to overcome intrinsic non-responsiveness to drugs), and the optimal clinical use of haematopoietic growth factors including thrombopoietin. Since trials addressing specific questions in the treatment of elderly patients with AML have remained scarce, the initiation of these studies is sorely needed. One may hope that these clinical trials will provide some of the necessary answers and new clues to advance future therapy of AML in the elderly.

References


Therapeutic approaches to the older patient with acute myeloid leukaemia

Bob Löwenberg
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Introduction
The majority of patients with acute myeloid leukaemia (AML) are 60 yrs of age or more. Although intensified cytotoxic therapy and bone marrow transplantation have improved the prognosis of AML in recent years in young and middle-aged adults, these therapy approaches have proved of only little benefit to individuals with AML over 60 years old, whose prognosis, therefore, is worse. The reasons for the different treatment outcome between young and middle-aged adults and older patients are probably multiple. Elderly people may suffer from AML which is a priori more resistant to chemotherapy. Furthermore, the ability of aged individuals to withstand the toxicity and morbidity caused by intensive chemotherapy is evidently far more limited.

Remission induction treatment
Remission induction chemotherapy usually involves a combination of the chemotherapeutic agents cytarabine and an anthracycline (daunomycin, idarubicin) or an anthracenedione (mitoxantrone). The prevailing opinion today is that intensive chemotherapy is the treatment of choice for patients of 60 yrs and over suffering from AML, if they are fit.1 Following one or two cycles of chemotherapy approximately 40-55% of them may enter complete remission. Thus, it is evident that the elderly population of patients respond significantly less favourably to induction treatment. The inverse relationship between age and complete remission rate is apparent from a number of studies that enrolled patients covering a broad range of ages. These results are indicative of a progressive decline of the response to chemotherapy with increasing age. How can these differences be explained? Are they due to differences of the dose or the choice of the chemotherapeutic agents? Or are they perhaps due to the occurrence of AML of greater risk in patients aged 60 yrs and over? Only a few prospective randomised studies have addressed the question of choice of drug and choice of dose and revealed differences in efficacy. Two studies addressed the question of the possible efficacy of mitoxantrone as compared to daunomycin, but did not provide an unequivocal answer. In one study2 mitoxantrone produced significantly better response rates in elderly patients. In contrast, in another study mitoxantrone and cytarabine as first-line therapy did not improve the likelihood of response.3 One recent phase III study involving more than 500 patients showed a slight advantage of mitoxantrone-cytarabine therapy (as compared to daunomycin-cytarabine treatment) with respect to response, but no difference with respect relapse or overall survival.4 A critical evaluation of the comparative value of idarubicin in older subjects has yet to be made.

Complications and mortality during remission induction therapy
Approximately 30% of patients who do not enter remission, fail due to early death or death during the hypoplastic phase post-chemotherapy. Death occurs during hospitalisation in spite of supportive care including antibiotics and red blood cell and platelet transfusions. The major cause of death among these individuals is infection. The greater death rate would indicate that elderly individuals are less able to tolerate the consequences of severe infections. They succumb more frequently to the negative effects on cardiac, pulmonary or renal function or haemodynamic dysfunction. The inability of elderly subjects to regenerate haematopoiesis in response to cytotoxic therapy or other perturbations, the reduced ability to regenerate marrow, the reduced tolerance of other organs is handling the toxic effects of drugs as well as the reduced elimination and metabolism of drugs might all explain why elderly patients are at increased risk of haematopoietic and other complications of intensive chemotherapy. The incidence of severe infections in elderly patients with AML on induction therapy is approximately 20%.5 If one considers the entire induction phase including the interval of post-chemotherapy hypoplasia approximately 20% of patients die.6 Apparently, a substantial proportion of over-60 years olds with AML do not survive remission induction therapy because of infectious or haemorrhagic complications during or following chemotherapy. Those who experience significant toxicity following the first cycle of chemotherapy are generally withdrawn from additional efforts at treatment. The obstacles to offering adequate therapy to older patients with AML are still, apparently, quite formidable. There is hope that haematopoietic growth factors may have a role in hastening haematopoietic recovery, reducing the incidence and severity of complications and ultimately reducing mortality.

Use of haematopoietic growth factors
The use of haematopoietic growth factors to accelerate haematopoietic recovery and prevent morbidity has attracted wide attention. Granulocyte...
This benefit translated into fewer days of antibiotic use, shorter with use of either cytokine in these studies. The results of some retrospective studies also suggest that elderly patients generally present more often with serious infections unresponsive to antimicrobial therapy, and that cytokines might be justified in patients with drug resistance in AML. The myelodysplastic syndromes are more often seen in elderly patients and are likely to contribute to the increased incidence of high risk AML in older subjects. AMs developing after progression from MDS generally respond poorly to chemotherapy, thus providing another explanation for the unsatisfactory response to treatment.

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References


Molecular pathogenesis of chronic myeloid leukaemia

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Chronic myeloid leukaemia (CML) is a clonal myeloproliferative disorder of the primitive haematopoietic stem cell. The disease was first described by Virchow in 18451 who introduced the term Weißes Blut (white blood, leukaemia). CML serves as a paradigm for the utility of molecular methods to diagnose malignancy and to monitor patient response to therapy since this entity was the first neoplasia known to be associated with a specific chromosomal rearrangement, the Philadelphia (Ph) translocation t(9;22)(q34;q11), and the presence of two chimeric genes, BCR-ABL on chromosome 22, and ABL-BCR on chromosome 9.2

The Ph translocation adds a telomeric segment of the ABL gene from the long arm of chromosome 9 to the centromeric part of the BCR gene on chromosome 22, forming two new genes, BCR-ABL on chromosome 22q- (Ph chromosome), and the reciprocal ABL-BCR on 9q+. The ABL gene contains 11 exons, spans over 230 kb and encodes a 145 kD non-receptor tyrosine kinase. The breakpoint in the ABL gene occurs usually within the large intron 1, which spans over 200 kb.

The chimeric BCR-ABL gene is transcribed into a 8.5 kb BCR-ABL messenger RNA (mRNA) and translated into a BCR-ABL protein. ABL-BCR mRNA is detectable in about 60% of cases, but not have any functional role in CML.

Depending on the breakpoint in the BCR gene, three main types of BCR-ABL can be formed. The typical BCR-ABL gene in >95% of Ph positive CML patients is derived from a disruption of the 5.8 kb major breakpoint cluster region (M-bcr) leading to chimeric mRNA molecules with b3a2 and/or b2a2 junctions, depending on the involvement of exon b3 in the fusion transcript. The final product of this genetic rearrangement is a 210 kD fusion protein, p210BCR-ABL.

A second breakpoint cluster region in the BCR gene has been identified in Ph positive acute lymphoblastic leukaemia (ALL).3 In 60% of Ph positive ALL and in sporadic cases of CM L the breakpoint is located in intron 1, within the so-called minor breakpoint cluster region (m-bcr). As a consequence, only BCR exon e1 is joined to ABL exon 2 (e1a2 junction). The result is the translation of a p190BCR-ABL protein. A third breakpoint location in the BCR gene has been detected 3' from the M-bcr region between exons e19 and e20 (micro breakpoint cluster region, µ-bcr) resulting in an e19a2 (c3a2) BCR-ABL transcript and a p230BCR-ABL protein.4

Inspection of the intron/exon structure of the normal BCR and ABL genes5 shows a large number of potential in frame BCR-ABL fusions. BCR exons e1, e6, e12 (b1), e13 (b2), e14 (b3), e19, and e20 are in frame with ABL exons a2 and a3, although joining of BCR exon e12 and ABL exon a2 would generate a stop codon at the point of fusion. The SH2 domain of ABL, which is encoded by exons a3 and a4, is essential for transformation,6 therefore, it is extremely unlikely that any BCR-ABL fusion lacking either of these exons would cause CML. Examples of rare BCR-ABL transcripts capable of inducing CML are transcripts lacking ABL exon 2 (b3a3, b2a3), e6a2 transcripts,7 and transcripts with incomplete exons due to genomic breakpoints within the exon (e8a2) (Figure 1).

Consequences of the BCR-ABL fusion

The central role of BCR-ABL in several pathways which lead to uncontrolled proliferation has been shown in vitro and in vivo by transfection and transplantation experiments in mice.8,9 Also, blocking expression or activity of BCR-ABL in CML cell lines by antisense oligonucleotides, ribozymes, or tyrosine kinase inhibitors, reverses the leukaemic phenotype.10,11 However, it is suggested that a clonal expansion precedes the Ph translocation.12

The normal cellular ABL protein belongs to the non-receptor tyrosine kinases. It is regulated by cellular and extracellular stimuli. The cell growth suppression of normal ABL requires tyrosine kinase activity, nuclear localisation and an intact SH2 domain.13 In contrast to ABL, BCR-ABL is located in the cytoplasm and displays deregulated, constitutive tyrosine kinase activity. It binds and phosphorylates proteins of the RAS signalling pathway including Ras-related Gap and Rac and the Grb-2 adaptor protein.

The ABL tyrosine kinase phosphorylates several tyrosines in BCR-ABL itself,14 as well as a number of cellular adaptor and signal proteins (e.g. Crkl, Cbl). In CML Crkl links BCR-ABL to several key molecules leading to leukaemic cell growth and/or inhibition of apoptosis.15 The ABL tyrosine kinase can also activate the Jak-Stat pathway contributing to the growth factor independence seen in cell lines containing the BCR-ABL gene.

The presence of BCR-ABL also affects the cell cytoskeleton. BCR-ABL tyrosine kinase phosphorylates cytoskeleton-associated proteins, including paxillin.
and focal adhesion kinase. Compared to ABL, BCR-ABL binds significantly better to F-actin.

Mutation or deletion of tyrosine 177 (Y177) of BCR prevents transformation of cell lines. Y177 is phosphorylated by the ABL tyrosine kinase and binds to the adaptor protein and Ras activator Grb-2. The phosphoserine-threonine domain is required for activation of the ABL tyrosine kinase function. In ABL, the SH2 domain, the SH1 tyrosine kinase domain, and the F-actin binding domain are required for transformation (Figure 2).

The role of BCR-ABL in affecting adhesion is still controversial. β1-integrin function has been shown to be diminished in CML cells, and this observation could explain the impaired binding ability of CML cells to bone marrow stroma. In contrast, BCR-ABL may stimulate adhesion by enhanced expression of αβ integrins. Adhesion stimulates cell cycle progression by activation of cyclin A/cyclin dependent kinase 2 complexes and subsequent transition through the G1/S adhesion checkpoint.

Phenotypes of CML

It is not clear why in some circumstances BCR-ABL causes chronic and in others acute leukaemia. Since chronic phase CML is preferentially associated with the p210BCR-ABL and Ph positive ALL with p190BCR-ABL, different BCR-ABL proteins may determine the type of leukaemia. However, transfection experiments in mice did not show a strong correlation between type of transfected BCR-ABL and type of leukaemia. An explanation of the different phenotypes might be the higher transforming ability of p190BCR-ABL which may cause a transformation of the lymphoid cells originating from the stem cell as a consequence of the Ph translocation. To become fully transformed, the lymphoid lineage needs the occurrence of additional molecular lesions, must occur as happens during the lymphoid blast crisis of CML.

p190 CML is characterised by a significant monocytosis, variable basophilia, older age, a higher proportion of immature progenitors in blood and marrow and a more aggressive course of the disease in most patients.

A CM L variant (neutrophilic CML) has been associated with e19a2 BCR-ABL transcripts and with a benign clinical course of CML in most patients. However, even in this entity progression to blast crisis has been observed.

The BCR-ABL rearrangement derived from breakpoints in M-bcr, m-bcr, and μ-bcr will differ considerably in the number and integrity of BCR protein domains included in the BCR-ABL product. The impact of the individual domains on the phenotype of...
Molecular events in advanced disease

During the chronic phase of the disease, myeloid cells containing BCR-ABL retain the capacity to differentiate normally. Accelerated phase is an intermediate stage in which patients show signs of disease progression and become resistant to the chronic phase therapy. Eventually, there is progressive loss of the capacity for terminal differentiation resulting in terminal blast crisis. An increase of the BCR-ABL mRNA expression in the leukemic cell, in part due to duplication of the Ph chromosome, precedes the phenotypic transformation of the malignant clone.

Virtually all CML patients with p210BCR-ABL disease express p190BCR-ABL at low levels at diagnosis, probably as a result of alternative splicing of the primary RNA transcript. Rising levels of total BCR-ABL mRNA in sequential samples from patients who are not in cytogenetic remission may precede disease progression. In patients with p210BCR-ABL positive CML, p190BCR-ABL is not increased with evolution to acute phase.

Methylation of the proximal promoter of ABL is a common epigenetic alteration associated with clinical progression of CML. Specific methylation of the Ph-associated ABL allele accompanies clonal evolution in CML. There is evidence that the length of chromosomal telomeres might be associated with disease progression. Loss of p53 function due to mutations and/or deletions, has been implicated in the induction of blast crisis.

Molecular diagnosis of CML

Qualitative molecular methods to detect the BCR-ABL gene rearrangement or its products are southern blot analysis at the level of DNA, western blot at the level of proteins, or fluorescence in situ hybridisation (FISH). For diagnostic samples, the use of multiplex PCR has been suggested to detect simultaneously all types of BCR-ABL and BCR transcripts as internal controls in one reaction by using three BCR- and one ABL primer. This method allows the reliable detection of typical BCR-ABL transcripts, such as b2a2 or b3a2, and atypical types, e.g. transcripts lacking ABL exon a2 (b2a3 and b3a3) or transcripts resulting from BCR breakpoints outside M-bcr, such as e1a2 and e6a2.

Detection of minimal residual disease

In 1989, first encouraging results concerning detection of minimal residual disease (MRD) by polymerase chain reaction (PCR) in CML patients after allogeneic bone marrow transplantation were reported. However, conflicting data from a comparative multicentre study revealed serious problems in the method with a high rate of false positive results. Over the past ten years, PCR has been developed and optimised. Specificity has been considerably increased by the partial standardisation of methodology and the introduction of rigorous precautions to avoid contamination.

Since patients with leukaemia at presentation or relapse usually have a total burden of more than 10^{12} malignant cells, cytogenetics, western blot, and conventional FISH have a maximum of sensitivity 1%. A patient with negative results may harbour as few as zero or as many as 10^{10} residual leukaemic cells. At this point, the patient is judged to be in clinical and haematological remission.

Reverse transcription (RT)-PCR for BCR-ABL mRNA is by far the most sensitive assay in this context and can detect a single leukaemic cell in a background of 10^{9}-10^{6} normal cells. Therefore, PCR is up to four orders of magnitude more sensitive than conventional methods. However, patients who have no residual disease detectable by this technique may still harbour up to a million malignant cells that could contribute to subsequent relapse. The sensitivity with which residual disease can be detected will be limited by the amount of peripheral blood or bone marrow that can be analysed.

In view of the very limited value of qualitative PCR for clinical decision making, several groups have developed quantitative PCR assays to estimate the amount of residual disease in positive specimens. Most groups have initially used competitive PCR strategies that can effectively control for variations in amplification efficiency and reaction kinetics. In order to standardise results for both quality and quantity of blood, RNA, and cDNA quantification of transcripts of normal housekeeping genes, such as ABL or glucose-6-phosphate dehydrogenase (G6PD), PCR has been employed. The standardised results are expressed as the ratios BCR-ABL/G6PD, BCR-ABL/G6PD.

After allogeneic bone marrow transplantation (BMTr), serial quantitative RT-PCR analysis of peripheral blood specimens can effectively distinguish those patients who are destined to remain in remission from those who are destined to relapse. Patients who remain in remission after BMTr have persistently undetectable, low, or falling BCR-ABL levels on sequential analysis. After 6 to 9 months, BCR-ABL is not detectable in more than 95% of patients.
undetectable in most cases and remains so indefinitely. Other patients may remain intermittently or persistently positive for prolonged periods of time without evidence of cytogenetic relapse. The level of detectable BCR-ABL transcripts in these individuals is usually very low. In contrast, for patients destined to relapse, increasing or persistently high levels of BCR-ABL mRNA can be detected on sequential analysis, often several months before the cytogenetic detection of the Ph chromosome in bone marrow metaphases. The recognition of relapse at the molecular level provides a window for therapeutic intervention while the burden of disease is still relatively low. Patients who meet the criteria for molecular relapse receive treatment by donor lymphocyte infusions (DLI), if possible before the onset of haematological relapse, because early use of DLI is more effective than delayed treatment. The great majority of patients who respond to donor lymphocyte infusions achieve durable molecular remission (RT-PCR negativity). After treatment with IFN-α, virtually all patients remain RT-PCR positive despite the fact that many achieve complete cytogenetic remission. For patients in continuing cytogenetic remission, quantitative RT-PCR has demonstrated that the levels of detectable residual disease may vary between patients by as much as 10,000-fold. The actual level of residual BCR-ABL transcripts is related to the probability of relapse, opening up the possibility that molecular monitoring may identify a subset of patients from whom treatment may be safely withdrawn. The finding of long-lasting cytogenetic remission in some CML patients treated with IFN raises the question of whether this agent actually cures the disease.

Quantitative RT-PCR for BCR-ABL has been shown to be a reliable method for monitoring residual leukaemia load in mobilised peripheral blood stem cells, particularly in Ph-negative collections. Quantitative RT-PCR allows selection of the best available collections for reinfusion into patients after myeloablative therapy (autografting).

Recently, novel real-time PCR procedures using the ABI Prism (TaqMan) or LightCycler system have been developed that promise to simplify existing protocols. They also offer a unique opportunity to standardize the assay and to develop rigorous standards and controls. The assays are based on the concept of fluorescence resonance energy transfer (FRET) between two fluorophores and real-time fluorescence measurement according to the accumulation of PCR product cycle by cycle. Quantitative RT-PCR will shortly become a routine and robust basis for clinical decision making in CML.

Using an optimised RT-PCR method, BCR-ABL mRNA can be detected at a very low level of 1 to 10 copies per 10^6 cells in normal individuals with an age dependent frequency (4506, 6043). One interpretation of this finding could be that BCR-ABL, and probably several other fusion genes, are being continuously formed in mitotic cells in the normal bone marrow, but only the combination of the correct BCR-ABL fusion in the correct primitive haematopoietic progenitor has a selective advantage and becomes functional as an expanding clone (Figure 3). Therapeutic implications Examples of therapeutic strategies directed against the BCR-ABL signalling pathways are:

1. decrease or stop BCR-ABL transcription using antisense oligonucleotides or ribozymes;
2. inhibit the activity of BCR-ABL tyrosine kinase;
3. eliminate leukaemia cells using antibodies to BCR-ABL encoded proteins.

The most promising approach is the use of a new rationally designed compound, STI571, a 2-phenylaminopyrimidine derivative, which has been shown to selectively inhibit the tyrosine kinase of ABL and BCR-ABL at in vitro, cellular and in vivo levels. The drug selectively suppresses the growth of CFU-GM and BFU-E derived from CML, reduces the proportion of BCR-ABL positive LTC-IC and initiates apoptosis without inducing differentiation. After demonstrating promising results in phase I clinical trials, phase II studies in interferon resistant or intolerant CML patients are underway.

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References


The use of interferon-α with or without cytarabine and other new agents for the treatment of chronic myeloid leukemia

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C hronic myelogenous leukaemia (CML) is a monoclonal myeloproliferative disease associated with an acquired specific reciprocal chromosomal translocation which results in the formation of the Philadelphia chromosome (Ph: t(9;22)). This disease begins with a chronic phase which is generally insidious. The chronic phase, characterised by myeloid hyperplasia of myeloid cells with maturation capacity, is easily controlled by standard chemotherapy such as busulphan or hydroxycurea. These agents, however, do not suppress the Philadelphia positive haematopoiesis. Four drugs [interferon, cytarabine, homoharringtonine and an Abl tyrosine kinase inhibitor (STI 571)] have been introduced for the treatment of chronic phase CML, the most recent one, STI 571, displaying a strong anti-leukaemic activity. These compounds produce cytogenetic responses even when they are used alone, a result which might markedly influence the survival of the patients.

Interferons (IFN) are cellular glycoproteins with antiproliferative, antiviral and immunoregulatory properties. IFN-α, the molecule used in CML, was initially prepared from human leukocytes but is now essentially produced by recombinant techniques. It was the first non-myelotoxic drug to cause a marked reduction in Ph positivity in some patients. Because of its in vitro activity, cytosine-arabinoside (Ara-C) alone has been tested in vivo for the treatment of patients with CML in chronic phase.1 Treatment of two patients in chronic phase CML with low-dose Ara-C (s.c. continuous infusion) resulted in a significant reduction of Ph+ metaphases.2 Ara-C was administered by subcutaneous infusion at a dose of 20 mg/m2/day with the aim of inducing and maintaining a granulocyte count of < 2 000/µL for at least 10 days. After 2 or 3 successive cycles of Ara-c, the metaphases decreased from being 100% Ph+ to being 2/27 and 3/26 Ph+ in patient 1 and 2, respectively. In contrast, subsequent intensive chemotherapy with high dose hydroxyurea at a dose sufficient to maintain granulocytopenia and normal serum B12 levels reduced the Ph positivity to only 46% and 72%. Thus it was concluded that a selective anti-leukaemic effect was achieved by Ara-C but not by hydroxyurea. Also it was assumed that Ara-C has significant antileukaemic activity while hydroxyurea is a non-specific inhibitor of granulopoiesis. Subcutaneous cycles of Ara-C were also administered to 5 patients in chronic phase disease.3 Haematological remission was consistently observed and, in addition, 4 patients had a cytogenetic response. These results indicated that prolonged continuous administration of Ara-C could inhibit Ph-positive haematopoiesis. However, this inhibition was, in all cases, transient. The effects of IFN-α combined with Ara-C on leukaemic human haematopoietic progenitors cells has been investigated in vitro. This combination resulted in a stronger cytotoxic effect.4 Because of the different mechanisms of action of these two molecules, it has been suggested that treatment of patients with chronic phase CML with these two agents could result in more cytogenetic responses and better survival.

Homoharringtonine (HHT) is one of a group of cephalotoxin esters isolated in 1969. Homoharringtonine, a plant alkaloid, has been recognised as being an antitumour agent in Chinese folk medicine. Preclinical studies indicated good antitumour activity in murine P388 lymphocytic leukaemia and L-615 leukaemia.5 Several phase I-II studies documented some degrees of antileukaemia activity in refractory acute myelogenous leukaemia. The use of homoharringtonine in CML was first reported by Chinese teams. Of 15 patients in chronic phase, 9 achieved a haematological remission with a dose of HHT of 5 to 7 mg/daily for 7 to 10 days.6 However no cytogenetic evaluation was reported. The most important antileukaemia activity of HHT is that of inhibition of protein synthesis. However induction of apoptosis has also been demonstrated. In vitro HHT can produce dose-dependent cell growth inhibition. HHT exerted a synergistic effect with IFN-α, Ara-C and IFN-α+Ara-c in inhibiting CML haematopoietic progenitors. Such in vitro synergism is the rationale for clinical trials using the 3 molecules.7

The bcr-abl oncprotein plays a major role in the pathophysiology of CML. The protein kinase activity is also essential to the transforming function of bcr-abl. Thus bcr-abl tyrosine kinase is a logical target for protein kinase inhibitors. The 2-phenylaminopyrimidines were initially reported as being protein kinase inhibitors selective for the Abl and PDGF-R tyrosine kinase.8 One of these inhibitors (CGP 571 48B or STI 571) was potentially attractive because of its activity on v-Abl and PDGF-R kinases. In normal cells STI 571 inhibited autophosphorylation of v-Abl, PDGF receptor and Kit receptor. This compound also blocked the formation of PDGF-induced inosi-
tol phosphates, MAP kinase activation and c-fos mRNA expression. In colony-forming assays of peripheral blood or bone marrow from patients with CML, STI 571 induced a selective inhibition of the proliferation of Bcr-Abl expressing cells without inhibition of normal cells. However, using a single agent could result in leukaemia resistance. Thus, as for the other drugs, a combination of STI 571 with other antileukaemic agents has been evaluated for activity against Bcr-Abl positive cells. In vitro a combination of STI and IFN-α showed additive anti-proliferative activity against Bcr-Abl positive cells lines. However the most substantial inhibition was achieved with the combination of Ara-C and STI 571.

**Therapeutic results**

Interferon alone

The use of IFN-α in CML has been evaluated by the Houston group over the past 15 years. This group first used human leukocyte IFN-α in 51 patients in chronic phase treated with doses of 3-9 mega units daily. IFNs resulted in satisfactory control of the disease in 36 patients (71%) with normalisation of peripheral blood counts and a gradual decrease in bone marrow cellularity. Of greatest interest was the finding that serial cytogenetic studies showed a reduction in the percentage of Ph positive cells. Later, using recombinant IFN-α2a or 2b or 2c several studies from single institutions or co-operative groups confirmed the efficacy of IFN-α in CML. The dose of 5 x 10⁶ IU/m² daily was used at the MD Anderson Cancer Center for the treatment of 274 patients with early chronic phase CML. Of these 80% achieved complete haematological response (CHR) and 58% had a cytogenetic response (complete 26%, major 38%). The median survival was 89 months. Achieving a cytogenetic response after 12 months of therapy was associated with a statistically longer survival.

The Cancer and Leukemia Group B (CALGB) studied 112 patients, all newly diagnosed and previously untreated. Patients were treated with IFN-α2b at 5 x 10⁶ IU/m² s.c. daily. Among 107 evaluable patients, 63 (59%) obtained haematological responses with a median duration of 52 months. Of 80 patients for whom there were cytogenetic follow-up data, 17 (21%) achieved a partial cytogenetic response and 14 (18%) achieved a CCR (median time to response =12 months). In Schofield’s studies IFN-α was used at the dose of 2 MU/m² three times weekly. According to this report such a low dose was as effective as the higher dose schedule of 5 MU/m² daily which is the recommended dose. However the incidence and quality of the cytogenetic response were low. The relationships of the response to the dose and the schedule of the treatment by IFN-α, as well as the duration of chronic phase and survival were the subject of several randomised studies. In 1983 the German CML study group initiated a randomised study to compare the influence of hydroxyurea versus busulphan on the duration of chronic phase and survival in CML patients. By January 1991, 458 patients had been randomised, 226 to receive busulphan and 232 hydroxyurea. A third arm with IFN-α was opened later with an accrual of 164 patients. In this study, IFN-α was superior to BU therapy, but did not demonstrate a survival advantage after hydroxyurea. In October 1986, the United Kingdom Medical Research Council initiated a randomised trial comparing the intention to use or not to use human lymphoblastoid interferon, Welleferon, as maintenance therapy in CML in patients whose disease had been brought under control by either hydroxyurea or busulphan. In this trial major responses have been associated with interferon doses between 2 x 10⁶ and 6 x 10⁶ daily and responsiveness does not appear to be associated with higher doses. Out of 159 evaluable patients randomised in a Japanese trial, 80 received IFN-α. These patients achieved a significantly higher incidence of major cytogenetic responses than those receiving BU therapy. The predicted 5-year survival rates were 54% and 32% for the IFN-α and BU arms respectively. The Italian prospective comparative trial of IFN-α and conventional chemotherapy was recently updated. The observation period of living patients was between 95 and 129 months. Seventy-three of the 284 non-transplanted patients were alive, 56 (30%) in the IFN-α arm and 17 (18%) in the chemotherapy arm. In this trial, a low Sokal’s risk, haematological response and cytogenetic response were associated with a longer survival. However in a Benelux study the benefits of better haematological and cytogenetic responses after IFN-α did not translate into longer survival for the IFN-α treated patients. Survival, calculated from diagnosis, was no different in the IFN-α group (median 64 months) than in the control group (median 68 months). In order to establish whether patients may or may not benefit from treatment with IFN-α, a worldwide collaborative overview of the 1,554 patients randomised and assigned to treatment in 7 trials was conducted. The regimens that involved IFN-α produced a statistically significantly better survival than those involving either hydroxyurea (p=0.001) or busulphan (p=0.00007). The 5-year survival rates were 57% with IFN-α and 42% with chemotherapy. Patients achieving a complete or partial cytogenetic response had a significantly better survival than those obtaining CHR with minimal or no Ph suppression or those with partial haematological response or resistant disease. In addition most patients who develop durable major cytogenetic responses show some Ph suppression after 6 months of IFN-α therapy and Ph suppression to fewer than 50% Ph positive metaphases after 12 months of treatment. Responses to IFN-α were associated with several features, including phase of the disease, prior therapy, duration of chronic phase and IFN-α dose schedule. In several studies haematological and cytogenetic responses and response rates were better in patients with low risk (scoring system or Sokal index), compared with intermediate, or high risk diseases. The dosage schedule of IFN-α is critical for achieving haematological and cytogenetic responses. Patients receiving lower doses of IFN-α (between 6 x 10⁶ and 2 x 10⁶ IU/m², 3 times a week), had very low CHR rates and no evidence of Ph suppression. In contrast, protocols using 5 x 10⁶ IU/m² daily produced better haematological and cytogenetic responses rates. A higher incidence of major
(partial and complete) cytogenetic responses were also seen in patients with leukocyte counts above 3x10^3 μL and platelets counts above 100x10^3 μL.

Interferon and cytarabine

Combinations of IFN-α and chemotherapy have been given in order to achieve CHR or cytogenetic responses in IFN-α resistant patients. It was also assumed that a higher cytogenetic response rate might be achieved by increasing myelosuppression with a combination of myelotoxic drugs.

The combination of IFN-α and low dose Ara-C has been studied by several investigators. Since 1986 we have conducted 3 successive trials. In a first pilot study, 24 patients received hydroxyurea 50 mg/kg/day and IFN-α2a at a starting dose of 5x10^6 IU/m^2/d. Courses of low dose Ara-C were given to 11 patients at a dose of 10 to 20 mg/m^2/d for 10 to 15 days per month. A CHR was obtained in 18 patients, and 8 achieved a major cytogenetic response. A rapid cytogenetic improvement was recorded in 6 out of 11 patients receiving low dose Ara-C, with complete Ph chromosome suppression in 4. Two French multicentre trials were then conducted in order to study the potential benefit of a combination of IFN-α with low dose Ara-C as front line treatment. In the CML 88 study, all patients received subcutaneous IFN-α2b, starting at a dose of 5x10^6 IU, plus hydroxyurea (HU), 50 mg/kg/d orally until stable CHR was achieved. The maintenance treatment was scheduled to begin during the third month. Patients were randomised to receive either IFN-α2b alone or the same dose of IFN-α2b plus monthly courses of Ara-C at a dose of 10 mg/m^2/d, for 10 days. From April 1988 to January 1991, 237 patients were registered. A recent analysis of this trial was performed on 207 evaluable patients, 104 of whom were in the IFN-α2b alone group and 103 in the IFN-α2b and Ara-C group. The median follow-up is 85 months. In the IFN-α2b+Ara-C group, 29 of 103 patients (28%) achieved a CCR whereas 21 of 104 patients (20%) treated with IFN obtained this result; the median survivals are 77 months in the IFN-α2b group (20%) treated with IFN obtained this result; and 65 months in the IFN-α2b+Ara-C group. The median follow-up was 43 months in the IFN-α2b group (the differences are not significant). In each group, patients who achieved a major (<35% Ph+ cells) or a CCR had a significantly better survival than those with minor or no response. In the CML 91 study, the dose of Ara-C was increased to 20 mg/m^2/d, the monthly courses being started within 2 weeks after randomisation. The trial enrolled 810 patients, and 721 were studied: 360 randomly assigned to the IFN-α2b+Ara-C group and 361 to the IFN-α2b group. An update of this trial shows that patients in the IFN-α2b+Ara-C group survived significantly longer than those in the IFN-α group (p = 0.02). At 5 years the estimated survival rates were 70% (64-76%) in the IFN-α2b+Ara-C group and 58% (51-65%) in the IFN-α2b group. The median follow-up was 43 months (range 2-82). In this trial a relationship was also noted between cytogenetic responses and survival: the probability of having a major cytogenetic response at 24 months was significantly higher in the IFN-α2b+Ara-C group (p = 0.006). The rate of CHR was higher in the IFN-α2b+Ara-C group (66%) than in the IFN-α group (55%; p<0.01). Patients who received IFN-α2b and Ara-C had a higher probability of achieving major cytogenetic responses than those assigned to IFN-α2b (39% versus 26% p < 0.001). Major side effects were nausea, vomiting, diarrhoea and mucositis which were related to the use of Ara-C. Thrombocytopenia was also recorded with the combination. Severe episodes of depression led to discontinuation of IFN-α in both arms.

In 1992 the Houston Group reported results in patients with advanced stages of CML. Between 1987 and 1990, 60 patients were treated with a combination of IFN-α (5x10^6 U/m^2) and Ara-C (15 mg/m^2/daily) given by continuous subcutaneous infusion or in 2 subcutaneous doses for 2 weeks. Ara-C was administered until complete haematological remission was achieved and then given for 7 days every 4 weeks. IFN-α dosage was adapted to the white blood cell and platelets count. During maintenance Ara-C was given at a sufficient dose to achieve a granulocyte count of 10x10^3/μL and a lowest platelet count of above 60x10^3/μL. The results were compared with those in patients in similar CML phases but treated with IFN-α alone. Twenty-two of 40 patients (55%) in late chronic phase achieved CHR and partial plus minimal cytogenetic responses were observed in 15%. Better results were achieved in patients with shorter disease duration. The comparison with 39 the matched historical control patients showed advantages in the group of patients treated with the combination. The 3-year survival rate of patients treated with IFN-α plus Ara-C was 75%, which was better than the 48% in those treated with IFN-α alone. Of the 20 patients with accelerated phase disease, 10 achieved a CHR, four of whom (20%) achieved a cytogenetic response. Furthermore, 5 of 20 patients with cytogenetic clonal evolution, showed variable degrees of suppression of the additional clones. More recently, the same institution reported their results on the treatment of patients with early chronic phase CML. One hundred and forty patients received subcutaneous injections of IFN-α5x10^6 U/m^2 daily and a total daily dose of 10mg of Ara-C in the assumption that daily administration of Ara-C would be possibly less toxic and more effective. In this non-randomised trial, a CHR was achieved in 92% of patients and a cytogenetic response in 74% Major responses were noted in 50% and complete responses in 31%. After a median follow-up of 42 months, the 4-year estimated survival rate was 70%. The median time to obtain a major cytogenetic response was shorter with this combination than that achieved with historical IFN-α regimens. The Italian Co-operative Study Group on CML conducted a similar randomised trial. Five hundred and forty evaluable patients were randomised: 275 to IFN-α+Ara-C and 265 to IFN-α alone. At 12 months, the combination resulted in a higher cytogenetic response rate (41% versus 34%) and a higher major cytogenetic response rate (17% versus 9%; p=0.01). Survival was also improved by the combination.

Arthur and Ma reported a higher cytogenetic response rate in a group of 30 patients with early chronic phase CML whose diagnosis had been made less
than one year previously. Initial treatment with hydroxyurea was kept and adjusted in order to obtain a white blood cell count (WBC) between 1x10^9/L and 4x10^9/L. IFN-α was introduced at a dose of 3 million units daily and then escalated up to 9 million units daily. Ara-C was given at a dose of 20 mg/m² once daily for 21 days and repeated every 42 days. With this regimen a complete cytogenetic response was obtained in 30% of the patients and a partial response in 23%. Hence 16/30 (53%) patients achieved a major (complete + partial) cytogenetic response. An Austrian group also reported interesting results. Eighty-four patients were treated with IFN-γ2c at a daily dose of 3.5 MU in addition to monthly courses of Ara-C at a dose of 1 mg/m² for 10 days. A prephase with hydroxyurea was administered to produce an initial reduction of WBC to fewer than 20x10^9/L. In 45 patients (54%) complete haematological response was achieved and a cytogenetic response was recorded in 39 (46%) including complete responses in 15 (18%). In order to assess the efficacy and toxicity of a combination of IFN-α2b and Ara-C, IFN-α2b (5x10⁶ IU/m²) was combined with Ara-C at a fixed daily dose of 20 mg, for 14 days. No benefits from the addition of Ara-C were reported in this trial.

**New agents**

**Cytarabine Ocfosfate**

Cytarabine ocfosfate (YNKO1) is a lipophilic derivative of Ara-C, which is resistant to deoxycytidine deaminase and can be administered orally. The drug is absorbed from the intestine following oral intake, taken up into hepatocytes and then slowly metabolised to Ara-C plasma levels after oral administration of IFN-α. Ara-C plasma levels after 300 mg, the responses being observed within 3 weeks. At this dose level, 33% of the patients who received at least 10 mg of STI 571. Haematological responses were observed in all patients who received at least 140 mg of STI 571. Complete responses were recorded in 96% of the patients at a dose level of 300 mg, the responses being observed within 3 weeks. At this dose level, 33% of the patients achieved some degree of cytogenetic response with 2 patients displaying complete cytogenetic responses after 5 months of therapy. STI 571 is currently being tested in phase II trials for patients in myeloid blast crisis, or accelerated phase and in in interferon intolerant or resistant patients. Based on preliminary in vitro data it seems logical to investigate the potential of combinations such as STI 571 plus Ara-C or STI 571 plus IFN-α.

**Conclusions**

IFN-α is now considered as a major agent for the treatment of chronic phase CML. Although allogeneic bone marrow transplantation could cure more than 50% of patients, many can not be transplanted because of lack of a suitable donor. The good results of the combination of IFN-α and low doses of cytarabine have been confirmed by several randomised and non-randomised studies, thus supporting the use of IFN-α and low dose cytarabine as the best standard of care. Following initial treatment with a combination of IFN-α and Ara-C, patients who achieve CHR by 6 months and a major cytogenetic response (i.e. less than 35% Ph+) by 12 months may continue

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on therapy. It is expected that the survival for these patients could be prolonged. However homoharringtonine and STI 571 are emerging as promising new drugs. Side effects of these drugs are less frequent than with IFN-α. Studies are currently undergoing to investigate whether daily cytarabine administration (or its oral form, YNKO1) is better than intermittent administration, and whether combinations of HHT, IFN-α and Ara-C or STI 571+Ara-C or STI 571+IFN-α significantly prolong the survival of (or cure?) CML patients.

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References


Transplantation issues

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The management of chronic myeloid leukaemia (CML) has changed very greatly in the last twenty years. This is due mainly to the recognition that interferon-alpha (IFNα), either alone or in combination with cytarabine, can prolong survival in comparison with hydroxyurea, and to the fact that allogeneic stem cell transplantation (SCT) can cure selected patients. The definition of cure is always controversial. Nevertheless the observation that some patients with CML have survived 15 or more years after allografting and lack evidence of BCR-ABL transcripts at the molecular level is consistent with the notion that leukaemia has been permanently eradicated from their bodies. The observations do not, of course, exclude the possibility that residual leukaemia has been reduced to very low levels by the transplant procedure and that continuing control depends upon a continuing graft-versus-leukaemia (GvL) effect. Nonetheless these observations in allografted patients contrast with findings in patients who achieve cytogenetic remission after treatment with IFNα, the great majority of whom have persisting evidence of CML at the molecular level.

Because successful SCT is apparently the only curative approach for CML, but the risk of transplant-related mortality remains appreciable, the decision of whether and, if so, when to offer an allograft to a given patient is complex. It is rendered even more complicated by the recent introduction of STI-571, which could, in comparison with IFNα, prolong life and conceivably contribute to cure of CML. Moreover the approach of autografting with Ph-negative stem cells also offers the prospect of prolonging life and may indeed prove curative if current technology can be improved. I shall review here some of the controversial aspects of transplant procedures and focus on an approach that may help to guide the clinician in making a recommendation for a given patient. It must be stressed that the factors considered relevant to decision making in 2000 are likely to change greatly within the next five years.

Issues in allogenic-SCT

Timing of transplant
The optimal timing for an allogeneic-SCT (allo-SCT) procedure remains a matter of some debate. The results of allografting for patients whose disease has progressed to an advanced phase (accelerated phase or blastic transformation) are so comparatively poor that most experts are agreed that an allograft should be performed in chronic phase, if at all. The Seattle transplant group reported some years ago that the results of transplantation performed early after diagnosis were superior to those of transplantation performed later in the course of the disease. This was subsequently confirmed in an analysis reported by the International Bone Marrow Transplant Registry and accords with findings in individual series. The conclusion must, therefore, be that allo-SCT is best performed soon after diagnosis, certainly within one year, though it is still reasonable to offer an allo-SCT to a patient whose disease has been in chronic phase for longer.

There has been considerable debate as to whether the risk of transplant-related mortality (TRM) for a patient transplanted in chronic phase might be increased by previous treatment with IFNα. Some reports have suggested that this is indeed the case, while others found no adverse effects of previous exposure to IFNα. This is an important issue because one approach to decision making for the borderline patient is to offer a trial of IFN first and to assess response before attempting to make a decision whether or not to proceed to allo-SCT. A recent report from the German Cooperative Group for CML may resolve this issue. The group showed that the survival of patients previously treated with IFN but from whom the agent had been withdrawn 90 or more days before the allo-SCT, was identical to the survival of patients never previously treated with IFN; conversely IFN treatment within 90 days of the allo-SCT had an adverse impact on TRM.

Blood or marrow as source of stem cells
The introduction into clinical practice of G-CSF in the late 1980s made it possible to collect sufficient numbers of pluripotential stem cells (or more precisely CD34+ cells) from the peripheral blood to allow engraftment in the autologous setting. Subsequently it became clear that allografts could also be performed with stem cells or CD34+ cells mobilised into the peripheral blood of normal donors. In general collections of peripheral blood contain substantially more CD34+ cells and perhaps 10 times more lymphocytes than comparable collections of bone marrow. Recovery of neutrophil and platelet numbers is more rapid in recipients of blood-derived allogeneic stem cells than in those receiving marrow-derived stem cells. Conversely the incidence and severity of chronic graft-versus-host disease (GvHD) seem to be greater in recipients of blood-derived stem cells. The relapse rate after allografting for CML

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seems to be lower in patients who receive blood-derived stem cells.\textsuperscript{11} A number of prospective studies comparing clinical results of using blood or marrow stem cells are still in progress. For the present it seems reasonable to use marrow stem cells for the patient undergoing an allograft in chronic phase. One might speculate that for patients undergoing an allograft for CML in advanced phase, the use of peripheral blood stem cells would be preferable on account of the presumed increased potential of these cells for mediating a graft-versus-leukaemia effect. There is as yet no evidence in support of this speculation.

T-cell depleting of donor cells

Depletion of T-lymphocytes from the allogeneic marrow or blood is a highly effective method of abrogating graft-versus-host disease. Such depletion may be achieved by incubating blood or marrow cells with an anti-T or anti-lymphocyte polyclonal antibody or by intravenous administration of the antibody to the patient at the time of the transplant. T-cell depletion has, however, a number of disadvantages. It increases the risk of non-engraftment, it delays immune reconstitution, and most importantly, increases the risk of relapse.\textsuperscript{12} This last complication can be counteracted by prophylactic or pre-emptive administration of donor-derived T-lymphocytes starting soon after the transplant procedure. Clinical results of the one published study suggest that this approach may indeed be valuable in the management of CML.\textsuperscript{13}

Defining alternative donors

If allo-SCT with HLA-identical sibling donors can cure selected patients with CML, it is logical to consider the role of alternative donors for allografting patients who lack suitable siblings. Such alternative donors include phenotypically HLA-matched or near-matched family members, phenotypically matched unrelated volunteers and phenotypically matched donors of cord blood stem cells. The definition of HLA-matching is more complicated for alternative donors than for siblings. It depends usually on the use of DNA-based techniques to characterise genes of both class I and class II categories (ie HLA A, B, C, DR, DQ and DP). Van Rood et al. have made a valuable contribution by suggesting a system for categorising the degree of match between patient and donor.\textsuperscript{14} When more than one donor appears suitable for an individual patient the assay of alloreactive cytotoxic T-lymphocyte precursors in the blood of the prospective donor can aid donor selection.\textsuperscript{15}

In general the results of allografting with stem cells from alternative donors are inferior to results of allografting comparable patients with stem cells from HLA-identical siblings.\textsuperscript{16,17} The best results were reported recently from the Seattle group.\textsuperscript{18} They showed that actuarial survival at 5 years was 57%. However, the incidence of GvHD appeared to be higher than what might have been expected following sibling transplants. The number of children with CML who are eligible for allograft procedures is small and thus for the present few CML patients can be considered as candidates for allografting with cord blood stem cells.\textsuperscript{19}

How to manage relapse

The incidence of relapse within the first 5 years after allo-SCT for CML in chronic phase ranges between 0 and 30% and depends in part on the details of the transplant procedure and the method employed for preventing GvHD. As mentioned above, T-cell depletion is associated with a greatly increased risk of relapse. Similarly patients who receive cyclosporin A plus methotrexate post-transplant have a higher incidence of relapse than those who receive cyclosporin A alone. Patients allografted in advanced phase CML have a higher incidence of relapse than those transplanted in chronic phase. The majority of relapses occur within the first four years post-allo-SCT but patients remain at risk of relapse for much longer, possibly indefinitely.

Relapse when it occurs usually proceeds in an orderly manner, starting with molecular evidence of disease and progressing thereafter to cytogenetic relapse and eventually to haematological relapse. The whole progression may take months or years. Occasionally molecular or cytogenetic relapses prove to be reversible. On rare occasions patients apparently in complete remission relapse directly to blastic phase disease.\textsuperscript{20}

For these reasons it seems logical to monitor patients indefinitely by molecular methods or by serial examination of marrow metaphases or by fluorescence in situ hybridisation of peripheral blood neutrophils. Patients who satisfy current criteria for relapse should then be considered for further therapy. Currently the choice of treatment for relapse includes administration of IFN-\alpha, use of donor lymphocyte infusions (DLI) or a second allo-SCT using the same donor. In practice the best approach is probably to administer T-lymphocytes collected from the original transplant donor. Whereas originally such DLI were given as a one-off or single bulk dose, this method was associated with a substantial risk of inducing GVHD or marrow aplasia.\textsuperscript{21,22} The modified approach introduced by the group in Sloan-Kettering (New York) whereby DLI were administered on an escalating dose schedule induces complete remission with equal reliability and with much reduced risks of GVHD and graft failure.\textsuperscript{23} This therefore is now the optimal approach to the management of relapse after allo-SCT.\textsuperscript{24} In due course it may emerge that the use of a non-myelosuppressive stem cell transplant or STI-571 is equivalent to or better than DLI.

Issues in auto-SCT

Does autografting prolong life? Theoretically the administration of high dose chemotherapy followed by autografting with stem cells collected at diagnosis could prolong the duration of chronic phase and thus the duration of a patient’s life by reducing the total leukaemic stem cell mass or by eradicating a population of stem cells that had already progressed some way towards advanced phase. Moreover because nucleated cells collected at diagnosis actually comprise a mixture of Ph-positive and Ph-negative progenitor cells, autografting may re-induce Ph-negative haemopoiesis in some cases and this too may contribute to prolongation of life. In practice a number

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of retrospective studies suggest that autografted patients may live longer than expected,
but none of these studies is totally convincing. Attention has therefore focused in recent years on attempts to collect a population of progenitor cells that is relatively free of Ph-positive progenitors. Currently the most promising approach is that pioneered by the clinical research group in Genoa and replicated by other groups on both sides of the Atlantic.

The Genoa protocol involves administration of a combination of cytotoxic drugs (idarubicin, cytarabine and etoposide) to patients in chronic phase followed by collection of nucleated cells from the peripheral blood during the recovery phase by use of a continuous flow blood cell separator. If patients are treated in this way soon after diagnosis, the majority of cells collected are Ph-negative and many collections are also negative by RT-PCR. These cells can be used for autografting and in some cases patients regain Ph-negative haemopoiesis which may be sustained for 2 years or longer. Studies are required to show whether this interesting approach to the management of CML can in fact prolong life.

Decision making for the newly diagnosed CML patient
The primary decision in terms of managing the newly diagnosed CML patient must involve a consideration of the advisability of allo-SCT. This is turn involves consideration of what the patient can expect to achieve with non-transplant therapy. Currently the best approach to treating a CML patient who is not eligible for allografting appears to be the combination of IFNα plus cytarabine initiated soon after diagnosis. This conclusion may, however, have to be modified within the next one or two years because STI-571, either alone or in combination with IFNα,

may prove to be better than IFN plus cytarabine. Furthermore there is evidence that staging a patient’s disease in accordance with the formula proposed by Sokal may predict response to interferon and possibly also to interferon plus cytarabine. The formula proposed recently by the German Co-operative Group may prove even more valuable than the Sokal equation, which was derived before the introduction of IFNα.

Once the clinician has made a rough estimate of the probability of survival with optimal conventional treatment, it is appropriate to consider the chance of cure and of survival for a given patient subjected to an allo-SCT soon after diagnosis. In this regard Gratwohl and colleagues have made a valuable contribution to decision-making by defining five principal prognostic factors for survival after allo-SCT. They allocated a score of 0, 1 or 2 to each of the five factors in accordance with the degree to which the influence of that factor was favourable or unfavourable for a particular patient (see Table 1). Thus for each factor in a given transplant procedure a patient could score 0 (most favourable) or 2 (least favourable).

They showed that the aggregate prognostic score cal-

Table 1. Risk score for individual transplant procedures as established by the European Group for Blood and Marrow Transplantation.

<table>
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<tr>
<th>Feature</th>
<th>Score</th>
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<tr>
<td>A. Donor type</td>
<td></td>
</tr>
<tr>
<td>HLA-identical sibling</td>
<td>0</td>
</tr>
<tr>
<td>Unrelated/non-identical</td>
<td>1</td>
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Figure 1 - Survival of patients with CML whose clinical data were reported to the European Group for Blood and Marrow Transplantation according to risk score calculated on the basis of factors listed in Table 1. Reproduced from Gratwohl et al (32) with permission of the authors and publishers.
culated in this way correlated well with actual survival (Figure 1). This approach is extremely useful for helping a clinician and a patient in the clinic decide on the advisability of a transplant. It must be conceded, however, that many of the considerations in relation to deciding whether or not to proceed to transplant are strictly 'empirical'. There is a complete absence of prospective studies addressing the results of allo-SCT in CML - a point stressed in the recent report from the American Society of Hematology Committee on Practice Guidelines.

For the last five years at the Hammersmith Hospital in London we have used a treatment scheme or algorithm intended to guide the clinician and to a certain extent the patient in making a decision as to whether to proceed to allografting or to choose alternative therapies. At one stage patients were classified as good risk or poor risk for immediate allografting. Patients in the intermediate group were offered a trial of interferon-alpha2b plus cytarabine; if they failed the trial, they then proceeded to allografting. More recently this approach has been abandoned in favour of an approach whereby an attempt is made, on the basis of criteria specified above, to decide whether a patient should or should not proceed to allografting within 12 months of diagnosis (see Figure 2). This approach too may have to be modified if the early promise shown by STI-571 is maintained. STI-571 could also prove valuable for in vitro or in vivo purging of CML blood or marrow preparatory to autografting with Ph-negative stem cells.

References

chronic myelogenous leukemia does not affect outcome adversely provided it is discontinued at least 90 days before the procedure. Blood 1999; 94:3668-77.


14. van Rood JJ, Oudshoorn M. An HLA matched donor? What do you mean by an HLA matched donor? Bone Marrow Transplantation 1998, 22, Supplement 1, S83.


Understanding the pathogenesis of myelodysplastic syndromes

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It has been known for about 20 years that the myelodysplastic syndromes (MDS) comprise a distinct, albeit heterogeneous, group of haematopoietic disorders characterised by monoclonality and bone marrow failure. Whereas the monoclonal nature of MDS is responsible for the increased risk for leukemic transformation, marrow failure occurs because of aberrant progenitor proliferation and decreased survival resulting in cytopenia in the peripheral blood. During the last few years, major progress has been made in the understanding of the specific pathological processes leading to monoclonality, malignancy and marrow failure in MDS. These new scientific insights will be discussed in detail.

Monoclonality in MDS
The monoclonal nature of MDS has been clearly proven by cytogenetics, X-chromosome inactivation (XCI) studies and molecular methods for detecting mutations in oncogenes and tumour suppressor genes.

Cytogenetics
Non-random karyotypic abnormalities can be detected in 50-70% of patients with primary (p-) MDS and in 90% or more of therapy-related (t-) MDS myelodysplasia. These chromosomal aberrations vary from single numerical or structural changes to complex genomic lesions involving at least 3 different chromosomes. Whereas single chromosomal abnormalities occur more frequently in p-MDS and in an early stage of the disease, complex genomic changes are mostly detected in t-MDS and during disease progression.1 In contrast to primary AML, in which most chromosomal changes are balanced translocations, depletion of a part of a chromosome or whole chromosomal loss are amongst the most frequently encountered genomic changes in MDS. Partial or whole chromosomal deletions in MDS predominantly involve chromosomes 5 [del(5q), del(5q)], 7 [del(7q), -7], 20 [del(20q)], 11 [del(11q)], 12 [del(12p)], 13 [del(13q)] and 17 [del(17p)]. Other chromosomal changes frequently encountered in MDS are additional chromosomes (+8, +5, +6, +13, +21) but also reciprocal translocations, chromosomes 3, 5, 6, 8, 11, 12, 16 and 21 often being involved.1,2

The frequency of chromosomal abnormalities and more particularly deletions in MDS is thought to be related to genomic instability caused by an accumulation of genetic defects and/or the inability of the haematopoietic cell to repair DNA damage.3 Proof of extrinsic genomic damage is found in the chromosomal changes, including complex abnormalities and deletions of all or parts of chromosomes 5 and/or 7, in patients with MDS after exposure to genotoxic agents such as benzene and radiation, or after treatment with DNA-damaging chemotherapeutic agents.4 DNA and protein damage in MDS caused by increased oxygen free radical production has also been related to elevated plasma TNF-α concentrations5 and a reduced capacity to no metabolise genotoxins and oxidants normally. Glutathione (GSH) is one of the powerful antioxidant agents that mediate the inactivation of chemical toxins and carcinogens. Homozygous deletions of the gene encoding glutathione S-transferase theta (GSTT1) have been found more commonly in MDS than in controls.6 Additionally, mutations in the NADPH quinone oxireductase (NQO1) gene are related to a higher incidence of benzene-induced marrow damage.7

Besides reflecting genomic instability and being a disease-specific marker, chromosomal changes in MDS also have a prognostic value, they give information on progenitor cell involvement and they can help to unravel the molecular mechanisms leading to haematopoietic failure and leukaemic transformation.

The prognostic value of cytogenetics in MDS is exploited in the International Prognostic Scoring System (IPSS) according to which karyotypic changes have a major impact on survival and risk of leukaemic transformation.8 Regarding progenitor involvement, cytogenetics have clearly shown that clonal MDS mutations occur in the mature myelo-monocytic compartment but leave the lymphoid compartment, with the exception of only a few case reports of clonal B-cells.9,10 unaffected. This suggests that the cytogenetic mutations occur in a myeloid progenitor cell or in a progenitor common to the myeloid and B-lymphoid compartment. However, direct cytogenetic analysis at the progenitor level produces more heterogeneous data. Some investigators have not found the cytogenetic mutation in myeloid or lymphoid CD34+ progenitor fractions,11 whereas others have detected both primary and additional chromosomal changes at the level of immature CD34+lin- stem cells.12

Finally, important information is generated by the integration of the cytogenetic data with molecular investigations, in order to clarify how disruption of the...
normal structure and function of genes involved in MDS mutations affects progenitor survival, proliferation and differentiation. The most important known chromosomal regions and their corresponding candidate genes involved in MDS pathogenesis are summarised below.

Since del(5q) is the most common cytogenetic abnormality in MDS (10-15% of p-MDS, 50% of t-MDS) much effort has been spent in identifying the molecular structure and function of this deleted region. This has allowed the deletion to be narrowed down to 5q31-5q33 which includes several cytokine-genes (GM-CSF, IL-4, IL-5, IL-9), the FM S gene encoding the M-CSF receptor, and 2 genes (IRF1, EGR1) involved in signal transduction and transcriptional regulation. Chromosome 7 deletions are significantly associated with expression of the P170 MDR glycoprotein and the critically deleted region at 7q22 probably includes genes involved in DNA repair. Genes mutated at 12p13 are involved in cell cycle regulation and include cyclin-dependent kinase inhibitor (CDK1) p27, cyclin D2 and the ETS-related gene TEL/ETV6. Translocations of the MLL gene on 11q23 to chromosome 16 with its subsequent fusion to the transcriptional coactivator CBP (a CREB binding protein) and fusion of the AM L-1 gene on 21q22 with loci on chromosome 3 are mostly limited to t-MDS. Deletions of the short arm of chromosome 17 (the 17p- syndrome) represent a specific MDS subgroup with particular abnormalities in the granulopoietic lineage and frequent loss of the p53 tumour suppressor gene.

X-chromosome inactivation (XCI) in MDS

From the initial report published by Prchal in 1978 until now, clonality studies with XCI have, like the cytogenetic data, undoubtedly proven the monoclonal nature of myeloid-lineage derived cells, erythrocytes and platelets in the majority of MDS patients. However, using XCI-based techniques, the cytogenetic data, undoubtfully proven the monoclonal nature of myeloid-lineage derived cells, erythrocytes and platelets in the majority of MDS patients.21 This discrepancy can be explained in two ways. Firstly, monoclonal XCI patterns of lymphocytes can be due to constitutional or acquired skewing. Secondly, the possibility exists that due to their long life-span, the polyclonal T-lymphocyte population is part of a population that has originated before the onset of the clonal mutation in an immature progenitor. This implies also that the cytogenetic mutations represent a secondary clonal hit preferentially affecting myeloid progenitors and leaving the lymphoid compartment unaffected. Longitudinal follow-up of clonality of lymphocytes in MDS patients is required to confirm or reject this hypothesis. By using polymorphisms in the HUMARA gene, immature CD34+ and committed CD34+ progenitors were shown to be clonally mutated in high-risk MDS patients.22 Intensive chemotherapy regimens could restore polyclonal CD34+ and CD34+ haematopoiesis in some, but not all, of these patients, thus confirming the polyclonal nature of some haematological remissions in MDS.22,23

Other molecular changes in MDS

Mutations in codon 12 or 13 from the guanine-nucleotide-binding protein N- or K-RAS have been reported in a varying frequency in MDS with a mean of 30-40% for all FAB subtypes.3 RAS mutations can be detected in immature and committed precursors, preferentially in the CMML and carry an increased risk for leukaemic transformation. Mutations in the p53 tumour suppressor gene range from 0-23% in MDS and are associated with disease progression and a poor prognosis. Other cell cycle controlling genes, such as those encoding the p15, p16, p18 and p19 CDKI family members, are rarely mutated in MDS,24 but hypermethylation of p15 is frequent, particularly during disease progression. Finally, the role of increased expression in advanced MDS of the Wilms tumour gene (WT1), a transcriptional regulator of haematopoietic proliferation and differentiation, still needs to be determined.26

In summary, these clonality data confirm that cumulative genomic damage in the haematopoietic progenitor compartment is one of the key regulators of the phenotype and natural disease course in MDS. Early clonal events giving growth advantage to a (pre)malignant progenitor are followed by accumulation of other mutations triggering the leukaemic transformation. Whereas the total amount of genomic damage in MDS is probably underestimated, the critical amount of damage for monoclonal proliferation and the key regulators for transformation, are not yet fully identified. Newer techniques such as multicolour FISH and DNA screening, together with increasing knowledge about the role of individual genes, will allow us in the near future to find a common denominator in the cascade of genomic damage in this heterogeneous disease. This will not only result in better understanding of MDS, but also challenges to tailor newer molecular-based therapeutic strategies.

Marrow failure in MDS

Besides monoclonality, MDS haematopoiesis is also characterised by impaired progenitor proliferation and differentiation, and decreased cell survival. Haematopoiesis occurs in close proximity to a permissive micro-environment consisting of cellular elements, the extracellular matrix and, autocrine and paracrine acting cytokines. Apart from having an anchoring function, the stromal micro-environment also regulates progenitor growth and survival. Both the progenitors and the stromal micro-environment are dysfunctional in MDS.

Haematopoietic progenitors

It is now about ten years since it was established that MDS haematopoiesis is associated with increased levels of intramedullary apoptosis. Initial reports relying on morphological observations27,28 were confirmed by later studies using more sensitive molecular techniques.29 Excessive apoptosis occurs in all MDS FAB subtypes and affects the erythroid, myeloid and megakaryocytic lineages with frequencies between 17%30 and over 50%.31 These pronounced variations in the amount of intramedullary apoptosis can be, at least partially, explained by differences in the...
sample origin (marrow aspirates vs. bone biopsies), FAB subtype, time between sample procurement and analysis, and the sensitivity of the technique for detecting apoptosis. Most reports agree that the highest apoptotic rates occur in RA, RARS and RAEB, there being a progressive decline in apoptosis as the disease evolves towards RAEB-t and SAML. With disease evolution the phenotype of the apoptotic population can also shift from being preferentially CD34+ to CD34-. No correlation between rates of apoptosis and cytogenetics or monoclonality has been proven.

Excessive apoptosis in MDS may result from an increase in apoptosis-promoting and/or a decrease in survival-promoting cytokines, activation of the Fas-FasL system, and intrinsic cell cycle dysfunction.

Several authors have reported increased levels of TNF-α in serum and bone marrow in MDS. Levels of TNF-α and IL-1α correlate with the degree of apoptosis and TNF-α can induce apoptosis by oxidation of DNA and proteins.

Additionally, survival signals are frequently decreased in MDS, this is reflected by reduced levels of cytokines in serum and bone marrow. This balances the cell cycle towards programmed cell death or apoptosis. Confirmation of these in vitro data is found in the results of in vitro treatment of MDS patients with TNF-α blocking agents and with cytokines such as G-CSF and erythropoietin, with a subsequent reduction of apoptosis in responding patients.

Fas or CD95 is a 45 kD membrane protein that can transduce an apoptotic signal when cross-linked by its natural ligand FasL or by an agonistic anti-Fas antibody. Fas is upregulated by cytokines such as TNF-α and IFN-γ. Fas expression in MDS has been found to be increased on a variety of MDS marrow cells in triggering MDS apoptosis is still largely unknown. Stromal growth of MDS marrow is seriously impaired and is associated with a decreased capacity to support normal and myelodysplastic haemopoiesis. Increased numbers of stromal macrophages are the key regulators in the increased production of pro-apoptotic cytokines such as TNF-α, IFN-γ and TGF-β. Whether altered integrin-mediated focal contacts between MDS progenitors and marrow stroma are involved in apoptosis or in the increased proliferation rates reported to be frequently associated with apoptosis (antonymy), is at present unknown.

The above mentioned data summarise the current knowledge regarding the frequency, triggers and effectors of apoptosis in MDS.

Whatever the cause or mechanism leading to apoptosis, it is now generally accepted that increased inamedullary programmed cell death in MDS marrow is responsible for the cytopenias in the peripheral blood. However, apoptosis is not a feature unique to MDS. It is still, therefore, enigmatic whether increased apoptosis in MDS is related to the molecular pathogenesis of the disease, or whether it is only a consequence of ineffective haemopoiesis caused by genomic damage and micro-environmental changes. Finally, the possibility still exists that apoptosis in MDS is only an escape phenomenon aimed at eradicating the malignant clone. This would imply that medical efforts to block apoptosis in MDS might enhance leukaemic transformation.

Summarising the recent literature data, it becomes clear that the pathogenesis of myelodysplastic syndromes is complex since both haematopoietic progenitors and the haematopoietic micro-environment are involved in the disease process. However, during the last years, several groups have made important scientific contributions by describing recurrent abnormalities in the genotype and phenotype of MDS haemopoietis. The major challenge for the near future is to refine these observations and try to link the genomic damage in MDS with the impaired proliferation, differentiation and cell survival. Better and perhaps curative “MDS-tailored” therapeutic regimens.

Acknowledgements

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References

4. Karp JE, Smith MA. The molecular pathogenesis of treatment-induced (secondary) leukemias: founda-
Session 6 – MYELODYSPLASTIC SYNDROMES

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The myelodysplastic syndromes (MDS) comprise a heterogeneous group of clonal stem cell disorders characterised by ineffective haemopoiesis and a propensity towards leukaemic transformation. The elderly nature of the majority of patients with MDS, the overlap of these diseases with other neoplastic stem cell disorders and their variable clinical course, has created difficulties in designing appropriate therapies for such patients. A clearer understanding of the pathogenesis and biology of MDS, the identification of prognostic factors and development of novel agents should lead to a more risk-stratified and biologically targeted therapeutic approach.

**Therapeutic dilemmas in MDS**

A number of dilemmas exist regarding therapy in MDS, namely who, when and how to treat. The French, American, British (FAB) classification system, based on blood and marrow morphology, has contributed towards identifying prognostically discrete subsets of MDS. Variable clinical characteristics and survival within FAB subgroups, however, indicate that blast percentage alone is insufficient to assess risk accurately. The recently published International Prognostic Scoring System (IPSS), which incorporates marrow blast percentage, karyotype and clinical Prognostic Scoring System (IPSS), which in-...
counts, normal cytogenetics, low serum tumournecrosis factor-α (TNF-α) levels and increased mar-row erythroblasts. In contrast to therapy with EPO alone, response rates in RARS equalled those seen in other FAB subtypes. Physiologically, erythropoietin exerts its effects through inhibition of apoptosis and promotion of erythroid progenitor differentiation. Similarly, in MDS, response to G-CSF and EPO is associated with a significant reduction in bone marrow (BM) apoptotic cells (58.8% vs 44.5%, p = 0.0003) and decreased CD34 positive cell ratio of pro-apop-totic c-myc versus anti-apoptotic Bcl-2. Addition of the differentiating agent all-transretinoic acid (ATRA) may further reduce BM progenitor cell apoptosis. Concerns that the use of growth factors may pro-mote leukemic transformation have not been borne out in long-term studies.10

Trophic and differentiating agents
5-azacytidine (5-Aza) and 5-aza-2-deoxycytidine, pyrimidine analogues which inhibit DNA methyl-transferase activity, conceivably improve myelodys-plastic haemopoiesis by reversing aberrant gene methylation and permitting cellular differentiation. Trials of 5-Aza in MDS have, to date, been largely re-stricted to high-risk MDS patients. Nonetheless, up to 63% patients have demonstrated improvements in blood counts and/or cytogenetic responses. More-over, a large prospective randomised phase III study conducted by the Cancer and Leukemia Group B (CALGB) found that 5-Aza treated patients showed a significantly lower probability of AML transforma-tion (11% vs 31%, p = 0.003) and a longer median time to leukaemic progression or death compared to patients in the observation arm (22 months vs 12 months, p = 0.0034).13 Quality of life was also im-proved in patients receiving 5-Aza.14 The main side ef-fect of myelosuppression with 5-Aza however, may limit its application in patients with low risk MDS and brings into question whether its antileukaemic properties are derived through differentiation in-duction or through clonal suppression.

Sodium phenylbutyrate (PB), an aromatic short chain fatty acid, also has potent differentiating effects on leukaemia and solid tumour cell lines. Its precise mechanism of action is unknown although it appears to alter transcriptional regulation of gene expression through inhibition of DNA methylation and histone de-acetylation. In a phase I trial, 17/27 (63%) patients with MDS or non-proliferative AM L experienced improvements in neutrophil counts and three showed a rise in platelet counts.15 A phase II study is currently under way to further evaluate the efficacy of PB in MDS.

Amifostine, a pro-drug that is activated by mem-brane alkaline phosphatase to form the aminothiol, WR-1065, enhances in vitro growth of normal and MDS multipotent progenitors and erythroid bursts and reduces the proportion of BM CD34+ cells under-going apoptosis. The trophic effects of amifostine metabolites appear to be mediated through their structural resemblance to polyamines which bind DNA and regulate cellular proliferation and differentia-tion. Amifostine also attenuates bone marrow stromal secretion of the pro-apoptotic cytokines TNF-α and IL-1β. Initial phase I/II trials demonstrat-ing improved in vitro colony growth and single- or multi-lineage haematological responses in 15/18 pa-tients (83%) treated with amifostine,16 have not been confirmed in all studies.17

Low-intensity cytoreduction
Sustained myelodysplastic clonal suppression through use of low-intensity chemotherapy repre-sents an attractive therapeutic strategy in more advanced FAB MDS subtypes, especially in older pa-tients and those with pre-morbid conditions. Treat-ment with low-dose cytoreductive 5-Aza or topotecan has yielded response rates of 10-25% in patients with MDS or AM L-secondary to MDS. Predictors of response in-cluded normal pre-treatment platelet counts and normal karyotype. A large randomised trial, how-ev-er, has shown no survival benefit of LDAC therapy over supportive therapy alone.18 Addition of GM-CSF and IL-3 failed to improve response rates or survival. Similar results have been achieved with low-dose anthracyclines and homoharringtonine, a cephalotoxin ester with reported in vitro cytotoxic and differenti-ating activity. Therapy with low-dose oral melphalan has produced durable complete haematological re-sponses in one third of elderly patients with interme-diate-2 to high-risk MDS, especially in cases with nor-mal cytogenetics and a normo- to hypocellular mar-row prior to therapy.19 The topoisomerase I inhibitor topotecan, given as a five day continuous intravenous infusion, has yielded responses in 26/60 (43%) pa-tients with RAEB, RAEB-t or CMML, with nineteen pa-tients (32%) achieving haematological and cyto-genetic CR. Addition of cytarabine to the above reg-imen further improves response rates [CR, 48/86 (56%)] and appears to be particularly effective in pa-tients with karyotypic abnormalities involving chro-mosomes 5 and 7 (CR, 71%) or secondary MDS (CR, 72%).20

Modulation of apoptotic signalling pathways
A number of studies have indicated that excessive haemopoetic cell apoptosis and consequent cytope-nias in early MDS may be mediated, at least in part, by the inflammatory cytokines TNF-α, transforming growth factor-β (TGF-β) and interleukin 1b (IL-1b). Blockade of these cytokine signalling pathways, using a combination of the methylxanthine pentoxifylline (PTX) and ciprofloxacin which inhibits hepatic PTX metabolism with or without dexamethasone, signifi-cantly decreases BM TNF-α levels and improves cyto-penias in 18/51 (35%) MDS patients. Addition of the cytoprotective agent amifostine appears to improve response rates further (22/29, (76%) although no patient treated with this combination has yet achieved complete remission.21

Given that most death signals are executed through a common caspase cascade, the use of caspase in-hibitors represents an attractive mechanism whereby excessive apoptosis in early MDS might be amelio-rated. Indeed, addition of specific inhibitors of cas-pase-1 and -3 to MDS BM cultures significantly in-creases both myeloid and erythroid progenitor growth. Alterations in mitochondrial membrane poten-tial, which play a key role in apoptosis regulation,
may also be pharmacologically modulated. Thus, cyclosporin A and bongkrekic acid which block mitochondrial pore opening and subsequent PCD, may be useful in early MDS, whilst arsenic compounds may play a role in advanced disease through mitochondrial-induced inhibition of apoptosis.

Dysregulation of protein tyrosine kinase (PTK) activity, enzymes that play a major role in cellular proliferation and differentiation, has been implicated in a number of haematological malignancies. In MDS, aberrant PTK-induced signalling, arising through activating mutations of the receptor tyrosine kinases, platelet-derived growth factor-β (PDGFR-β), c-kit or flt-3 or the signalling proteins n- or k-ras, is frequently associated with disease progression. Inhibition of PTKs and the signalling pathways in which they participate, therefore offers a novel therapeutic approach in these disorders. Promising results with the PTK-inhibitor STI-571 in Philadelphia positive chronic granulocytic leukaemia (CGL) has encouraged trials of this drug in bcr;abl negative myeloid neoplasms such as CMML and atypical CML, disorders commonly associated with aberrant PTK-mediated signalling. The PTK inhibitors genisten and herbimycin A also appear to inhibit leukaemic growth both in vitro and in mouse models.

Molecules that block ras function through inhibition of ras farnesyl-geranylation, may also demonstrate useful anti-tumour effects in some forms of myelodysplasia. Preclinical studies indicate that these agents can suppress transformation and tumour growth in vitro and in animal models, with little toxicity to normal cells. Moreover, in juvenile myelomonocytic leukaemia, a disorder frequently associated with dysregulated ras signalling, farnesyltransferase inhibitors appear to inhibit in vitro granulocyte-macrophage colony growth in a dose-dependent manner.²²

Immunomodulatory Agents

The frequent overlap of some forms of myelodysplasia with the bone marrow failure syndromes AA and PNH, and the recognition that suppression of haemopoiesis may be immune-mediated, has prompted trials of immunomodulatory therapy in MDS. Treatment with anti-thymocyte globulin (ATG) or cyclosporin A has produced sustained haematological responses in 44% and 84% cases respectively, with 12-23% patients achieving complete trilineage recovery. Predictors of response include less than 5% BM blasts, BM hypocellularity and the presence of a PNH clone prior to therapy.²³,²⁴ Response to immunosuppressive therapy has been associated with direct stimulation of MDS haemopoietic stem cells and loss of lymphocyte-mediated inhibition of CFU-GM. As recently observed in AA, combined immunosuppressive regimens (ATG, cyclosporin A, prednisolone) with or without G-CSF may yield superior response rates. There has recently been a resurgence of interest in the use of thalidomide in a number of immune-mediated disorders, including graft-versus host disease. Certainly, its immunomodulatory, anti-angiogenic and anti-TNFα properties indicate that its application in MDS should be explored further.

Intensive chemotherapy

The recently proposed International Prognostic Scoring System has helped to identify patients in whom median survival is shorter than 1 year. Attempts to improve outcome in this high risk group, with intensive chemotherapy regimens has yielded response rates ranging between 15-64%. Lower overall CR rates in MDS compared to de novo AML may be due to older patient age, a higher incidence of unfavourable cytogenetic abnormalities, progenitor cell phenotype and increased multidrug resistance-related antigen expression. Chemotherapy-induced pancytopenia is frequently prolonged and opportunistic infections are relatively common. Factors predictive of response to chemotherapy include younger age, a diagnosis of RAEB or RAEB-t compared to other FAB subtypes, primary rather than secondary MDS and shorter pre-treatment disease duration. In contrast, the presence of a poor risk karyotype is associated with both reduced rates and duration of remission.²⁵ The perception that MDS is associated with a poor response to chemotherapy has led to some reluctance to treat such patients with intensive cytotoxic regimens. When matched for the aforementioned risk factors however, patients with RAEB and RAEB-t attain similar CR rates to those seen in de novo AML.²⁶ Clinical trials employing investigational chemotherapy (fludarabine + cytarabine (FA), FA with G-CSF (FLAG) or FLAG with idarubicin (FLAG-Ilda) and topotecan with high dose cytarabine) have also produced encouraging responses and may be of benefit to MDS patients with poor-risk features.²⁷ Addition of agents which modulate multi-drug resistance such as quinine or derivatives of cyclosporin may further improve response rates.²⁸

Progenitor cell (PC) transplantation

Autologous PC transplantation

High relapse rates, short remission duration and low rates of long-term survival following intensive chemotherapy, have prompted trials of high dose cytoreduction with autologous PC rescue in patients with intermediate to high risk MDS who are not eligible for allogeneic transplantation. Evaluation of 79 MDS patients undergoing autologous PC transplantation in first CR has demonstrated transplant-related mortality (TRM), two-year overall survival, disease-free survival (DFS) and relapse rates of 10% 39%, 34% and 64% respectively. Patients younger than 40 years had a significantly better DFS than older patients (39% vs 25%, p = 0.04). Compared to de novo AML, patients undergoing transplantation for MDS/ MDS-AML had a significantly lower 2-year DFS (51% vs 28%, p = 0.025) and higher relapse rates (40% vs 69%, p = 0.007).²⁹

Because MDS is a clonal stem cell disorder, concerns have been raised regarding the feasibility of harvesting adequate numbers of cells of sufficient quality to re-populate BM following high-dose chemotherapy. Certainly, at our institution, mobilisation of PC was successful (≥2×10⁶/kg CD34⁺ cells plus ≥10×10⁶/kg) in only 4/20 (20%) patients with MDS (3/12 following G-CSF alone; 1/8 during re-generation following chemotherapy).

²⁵ 5th Congress of the European Haematology Association - Educational Book
Allogeneic PC transplantation

To date, transplantation of allogeneic PC following myeloablative therapy is the only curative option available for patients with MDS. Reported DFS rates range from 35-56% in different studies. Between 17-28% patients experience relapse while 32-48% succumb to non-relapse events. Factors associated with improved outcome following transplantation include younger age, shorter disease duration, primary MDS, <5% BM blasts, good-risk cytogenetics and low IPSS score at time of transplantation. Transplantation using an unrelated donor is associated with increased non-relapse mortality. Lower relapse rates associated with enhanced ‘graft-versus-leukaemia’ effect however, results in DFS rates that are comparable to those obtained with sibling transplantation. The role of induction chemotherapy prior to transplantation in MDS remains controversial. A number of studies have demonstrated no survival benefit of pre-transplant cytoreduction in patients with advanced disease. Patients who achieve a partial or complete response, however, appear to be at lower risk of post-transplant relapse than those with refractory disease, indicating that induction chemotherapy should possibly be reserved for cases with a reasonable chance of attaining CR.

The generally elderly nature of MDS patients and their frequent co-morbid conditions, limits the use of allogeneic PC transplantation due to unacceptable transplant-related mortality. Non-myeloablative conditioning regimens, which utilise ‘graft-versus-leukaemia’ immunological mechanisms for leukaemia cell abrogation, are associated with considerably lower toxicity and should potentially broaden the applicability of allogeneic transplantation in MDS. We have recently evaluated the outcome of allogeneic PC transplantation in 26 MDS patients conditioned with standard myeloablative protocols and compared them to nine cases receiving non-myeloablative preparative regimens. Overall TRM was 16% and 22/35 (63%) patients remain alive in complete remission at a median of 302 days (range 19-2063 days) post-transplantation. Despite significantly increased age and more frequent co-morbid conditions, patients receiving non-myeloablative conditioning experienced a significantly delayed onset and reduced duration of marrow aplasia, less mucositis, fewer days of fever, and reduced antibiotic, amphotericin, opiate and TPN use. The incidence of acute and chronic graft-versus-host disease and other post-transplant complications was also significantly reduced. No patient died in the immediate post-transplant period and 8/9 (89%) patients remain alive in complete remission at a median of 143 days (range 20-270 days) post-transplant (Table 1).

Summary

There has been substantial progress in our understanding of the biology and natural history of MDS over the last decade. Identification of the molecular lesions underlying its pathogenesis should allow identification of patients with early disease, while refinement of prognostic scoring systems will facilitate a more risk-stratified therapeutic approach. Elucidation of genomic events leading to dysregulation of the balance between cell growth and cell death in MDS will also allow the design of biologically targeted therapies.

In early MDS, novel therapeutic strategies that target excessive apoptosis should alleviate symptoms of cytopenias and may alter the natural history of the disease. For patients with poor risk features, the development of less toxic non-myeloablative preparative regimens, which target disease through alloreactive responses, may provide a more risk-stratified therapeutic approach.
tive immune cells, should broaden the application of allogeneic progenitor cell transplantation in MDS and will, it is to be hoped, offer the prospect of long-term survival to a significant proportion of patients.

References

3. Yoshida, Y. Hypothesis: apoptosis may be the mechanism responsible for the premature intramedullary cell death in the myelodysplastic syndrome. Leukemia 1993; 7:144.
High dose therapy for myelodysplasia

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The poor survival of patients with intermediate or high risk myelodysplastic syndromes (MDS) has increased the demand for improved therapeutic strategies, including intensified cytotoxic treatment with or without stem cell support. Until now, only the transplantation of allogeneic stem cells has proven to be a truly curative treatment, but at the expense of considerable transplant related morbidity and mortality.

Therapeutic strategies in MDS have been inspired by either missionary approaches, i.e. converting (pre)malignant cells into normal behaviour, or by crusader tactics, i.e. destroying non-compliant elements at the expense of innocent bystanders. These approaches may now become complementary rather than conflicting with the advent of immunotherapy (minitransplants) and/or monoclonal antibodies (e.g. anti-CD33).

The elaboration of risk-adapted treatment algorithms has been much facilitated by the use of the International Prognostic Scoring System (IPSS), which uses marrow blast percentage, cytogenetic data and number of cytopenias to delineate low, intermediate (1 and 2) and high risk categories.

While not much controversy exists on the treatment options for the older low risk patient or the younger high risk patient, the IPSS has intensified the discussion around optimal treatment for younger low or intermediate, and older (50, 55, 60, 65, ...) intermediate and high risk patients.

Intensive cytotoxic treatment

AM-L type chemotherapy can induce complete remission (CR) rates varying from 40 to 60 %. CR rates in MDS are lower than those in de novo in acute myeloid leukemia (AML) patients, treated with similar or identical, mostly Ara-C containing regimens. Two effects account for this: prolonged cytopenia, leading to higher early death rates, and increased drug resistance, mostly mdr-P-glycoprotein related.

Factors predictive for reaching CR include younger age, RAEB or RAEB-T versus AM-L following MDS, primary versus therapy-related MDS, the absence of cytogenetic abnormalities and the type of anthracyclines (e.g. mitoxantrone or idarubicin). Maintaining remission after CR remains a major problem. The median duration of disease-free survival rarely exceeds 12 months. For patients achieving remission, age seems to become less important as a predictor of overall and disease-free survival. This may have much to do with initial performance status. Unfavourable cytogenetics are the major determinant for poor survival after intensive chemotherapy.

About 15 % of MDS-patients achieving either CR or partial remission (PR) after intensive chemotherapy, may expect more than 5 years survival; some of these patients may, in fact, will be cured (French MDS-follow-up data). Long-term survivors are more likely to have suffered from RAEB-T at diagnosis, with normal or favourable cytogenetic findings. Earlier treatment in the course of the disease confers a better prognosis.

This indicates that at least some patient categories – even some that only achieve PR – may benefit from intensive cytotoxic treatment.

At present it is unclear whether newer therapeutic regimens containing fludarabine or decitabine, or making use of monoclonal antibodies (anti-CD33) may give higher CR rates and more prolonged remission duration. The use of myeloid growth factors – G-CSF or GM-CSF – may decrease the duration of granulocytopenia, but does not seem to increase survival.

Allogeneic stem cell transplantation.

Allogeneic stem cell transplantation can lead to prolonged long-term disease-free survival and cure in a selected group of MDS patients. The results of allo transplant are best in younger patients with untreated or minimally treated RA, as well as stable RAEB. Results of registry analyses have underscored that the status of the underlying disease at transplant greatly influences survival. EBMT data disclosed disease-free survival rates of 46%, 35%, 27% and 0% at 5 years for, respectively, RA/RARS, RAEB, RAEB-T and sAML. A multivariate analysis showed that the presence of bone marrow blasts > 30% negatively correlated with overall survival. Patients achieving CR seem to do much better than patients refractory to aggressive chemotherapy, with very few of the latter surviving after BMT. Patients with MDS or AM-L, related to previous chemo-radiotherapy, generally fare worse, especially if no CR can be obtained with induction chemotherapy.

The Seattle team reported on 251 patients with a median follow-up of 3.7 years and showed an actuarial DFS rate of 41 % with a cumulative relapse rate of 17 %. The 3-year cumulative incidence of non-relapse mortality was 42 %. A multivariate analysis again pointed towards increased age, advanced disease status, poor cytogenetics and therapy related disease as negative risk factors for both early death and relapse.

The high to very high non-relapse mortality remains disappointing.
Moreover, most MDS-patients lack a suitable HLA-identical sibling donor. The results of transplants using alternative, partially matched family donors and phenotypically matched unrelated donors, remain unsatisfactory and display a staggering transplant mortality of about 50%. The probability of disease-free survival is only 18 to 30% depending largely on age and interval between diagnosis and transplant. Many investigators regard the high to very high allograft mortality rates as unacceptable.

Recommendations for allogeneic transplantation in MDS

For high and high intermediate-2 risk MDS patients who have a molecular compatible sibling or unrelated donor, allogeneic stem cell transplantation (bone marrow or peripheral blood derived) is the treatment of choice.

Best results with allo transplant are obtained in younger patients, early in the course of their disease, but precisely this subgroup (<60 years, RA or RARS) was shown by IPSS analysis to have a median survival of more than 11 years without treatment. IPSS low and intermediate 1, risk categories thus hardly form an indication for early transplant, unless complex unfavourable cytogenetics or a life-threatening single cytopenia is present.

Unrelated donor transplants are at present not indicated in this category. In the Seattle series on unrelated donor transplants the actuarial non-relapse mortality at 2 years for RA patients (n=20) was 46%. In Leuven it was 50%. According to IPSS, these patients, if left untreated, can be predicted to have a 50% chance of surviving beyond 11 years.

Moreover, if the total Seattle data on 241 transplanted patients were subjected to IPSS score evaluation, slightly more than half of their patients (n=145) belonged to intermediate-2 or high risk categories before transplant and had a disease-free survival of only 32 and 24% respectively. This included sibling transplants.

As stated above, long-term disease-free survival with chemotherapy alone in this category amounts to about 15%, with 16% of deaths in aplasia.

Therefore definite recommendations regarding the early use of allogeneic transplants in MDS, outside well controlled and randomised studies, cannot be made. Attempts at reducing the risk of TRM are underway. Substitution of supralethal conditioning regimens by less toxic, tolerance-inducing chemotherapy or low-dose TBI, supplemented with delayed donor lymphocyte infusions (mini-transplants and DLI) may improve outcome considerably. Preliminary results have proven the feasibility of this approach, but results are definitely inferior to those obtained in lymphoma. The availability of large numbers of allogeneic peripheral blood progenitor cells - partially or totally T-cell depleted, with or without add-backs - offers new opportunities for improved transplant technology.

Autologous stem cell transplantation

The feasibility of autologous bone marrow or stem cell transplantation in both intermediate and high risk MDS patients has now been proven. The Chronic Leukaemia Registry of the EBMT contains data on almost 200 patients autografted for MDS or secondary leukaemia. Data on 79 of those transplanted in first complete remission were published. The 2-year overall survival, disease-free survival and relapse rates were, respectively, 39%, 34% and 64%. Patients younger than 40 years had a significantly better DFS (39%) than patients older than 40 (25%). The large majority of these patients were treated for a secondary leukaemia or therapy related MDS, only 19 underwent auto BMT for a primary RAEB or RAEB-T. The survival of only the latter was slightly better (46%) than the whole group. The transplant-related mortality was lower than 10%.

These data must be interpreted with caution. Only patients in CR were included in this retrospective analysis, excluding those not recovering from or resistant to induction chemotherapy. Moreover no data are available on the number of patients with MDS in whom an adequate marrow harvest could not be performed (persistent hypoplasia, fibrosis, poor performance, etc.).

Preliminary results on the influence of cytogenetic data on the outcome of autotransplant confirm previous data on intensive chemotherapy, i.e. actuarial 2-year survival of 52% in patients with good or intermediate risk, versus 28% in the poor risk group.

Even in high risk MDS, sufficient PBPC (i.e. >10^10 CD34/kg or >10^6 CFU-GM/kg) can be collected in first CR after priming with chemotherapy and G-CSF. Reinfusion of these stem cells after myeloablative chemotherapy results in much faster and more complete recovery of granulocyte and platelet counts. Consistent with this rapid recovery, days of fever, need for parenteral antibiotics, empiric antifungal therapy, transfusions of red cells and platelets and total duration of hospitalisation are significantly decreased compared to those in a historical matched auto BMT group.

Early relapse rates are not different from auto-BMT, while direct transplant-related mortality is less prominent.

A major concern remains the possible contamination of the peripheral blood progenitor cells by clonal malignant cells.

Preliminary results seem to indicate that in early stage MDS (RA or RARS) polyclonal haematopoiesis remains present and that polyclonal progenitors can be harvested with growth factors in steady state. After intensive remission induction non-clonal, putative benign, CD34+ progenitors can even be isolated from high risk MDS patients.

The early results of PBPC transplantation indicate that survival (not DFS) figures may approach those achieved with allogeneic transplants and even surpass those of unrelated transplants, because of low TRM.

Relapse rates remain, however, a major concern however. Whether PBPC transplants will improve survival rates over conventional high dose consolidation is at present uncertain.

References

1. Anderson JE, Thomas ED. The Seattle experience with
bone marrow transplantation for myelodysplasia.


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Molecular genetics of acute lymphoblastic leukaemia

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Acute lymphoblastic leukaemia (ALL) is a biologically and clinically heterogeneous disease. This diversity has its origins in the different cell types that are involved and the specific molecular genetic alterations that represent the proximal causes of clonal expansion. These underlying features vary substantially in relation to patient age and may have independent prognostic value in the context of particular therapeutic protocols. They are, therefore, of diagnostic value, an aid to patient management and help explain the very different clinical outcomes in childhood ALL compared to the adult form.

Many of the predominant or consistent genetic alterations in both ALL and acute myeloid leukaemia (AML) involve chromosomal rearrangement, illegitimate recombination and the generation of in frame fusion genes. Genes encoding transcriptional regulators (MLL, AML1/ CBFα, TEL) are key players in these promiscuous associations (Figures 1 and 2) and a principal impact of fusion gene formation and transcriptional deregulation may be a block to differentiation. Different fusion gene partnerships are selectively associated with subtypes of lymphoid or myeloid leukaemia. The basis for this link is unclear but most probably indicates that optimal impact of individual fusion genes is cell context dependent.

Unravelling the functional impact of mutant genes in the context of particular cell types in leukaemia may provide an explanation of the pathogenesis of disease, and of the variable outcomes of treatment and may also lead to novel approaches to therapy. Molecular abnormalities in leukaemic cells also provide unique clonal markers for tracking the natural history of the disease which in turn complement epidemiological studies seeking to unravel aetiological pathways.

A key issue is to unravel the natural history or time frame of clonal evolution of leukaemia which, in young children or infants, is necessarily brief. When does the initiating mutation arise? How many mutations are required? The recognised consistent molecular chromosomal abnormalities in leukaemia are acquired or non-constitutive changes. Which of these are primary events and, given the short latency in infants, does initiation usually occur prenatally in the foetus?

Analysis of mutant fusion genes in identical twins with concordant ALL have provided some unique insight into the clonal origins of the disease. In infant ALL, the most common of such mutations is the MLL-AF4 gene. In older children in the 2-5 year peak with B cell precursor or common (c) ALL, the equivalent molecular aberration is a fusion of TEL and AML1 genes. These genes are known (from mouse knockout experiments) to function normally as critical regulators of gene transcription and cell differentiation in haemopoiesis. The chromosomal breaks that generate these gene fusions occur within clustered non-coding regions (introns) of the involved genes but within the duster region, each patient's leukaemia gene break is unique. Molecular probing for genotypic fusion sequences by polymerase chain reaction (PCR) show, for MLL-AF4 in infants (<2 years) and TEL-AML1 in twin children (aged 3 to 14 years), that in each pair of twins, the same unique (clonotypic) and non-constitutive break occurs. The only plausible explanation for this finding is a single cell origin of the fusion gene in one foetus in utero, probably as a leukaemia initiating mutation, followed by intraplacental metastasis of clonal progeny to the other twin. The modest level of leukaemia concordance in cALL (~5%) contrasts with that in infants (25-50%) and suggests that foetal initiation by gene fusion is insufficient for clinical development of cALL and that some post-natal exposure and additional genetic event(s) are required to promote evolution of the foetal pre-leukaemic clone to frank malignancy.

Direct evidence for a prenatal origin can only be derived from prospective or retrospective detection of leukaemia-specific molecular abnormalities in foetal or newborn samples. A PCR-based method has been developed to scrutinise neonatal blood spots (Guthrie cards) for the presence of infrequent leukaemic cells at birth in individuals who subsequently developed leukaemia. Unique or clonotypic MLL-AF4 genomic fusion sequences are present in neonatal blood spots from individuals diagnosed with acute lymphoblastic leukaemia at ages 5 months to 2 years and therefore have arisen during foetal haemopoiesis in utero. This result provides unequivocal evidence for a pre-natal initiation of acute leukaemia in young patients. The most frequent translocation in older children with cALL is the t(12;21) TEL-AML1 fusion. A long range inverse PCR method has been developed to identify and characterise TEL-AML1 genomic fusion sequences rapidly and to design primers for analysing Guthrie cards from these patients. These latest studies now provide the first direct evidence for a prenatal origin of childhood common ALL in the peak age range of 2-5 years.

The combined twin and Guthrie card studies sup-
port a two-step aetiological model for childhood common ALL. Recent epidemiological studies provide some evidence that an abnormal reaction to common infections may, as predicted, provide the critical post-natal promotion step. The most frequent secondary genetic event in paediatric cALL is deletion of the non-rearranged TEL allele and this may be the rate-limiting step in the pathogenesis of cALL that requires aetiological exposure and promotion. For infant ALL with MLL gene fusions, the high concordance rate and remarkably brief latency suggests that an in frame MLL-AF4 (or ENL) fusion may be sufficient to produce leukaemia and that either no other genetic events are required, or, that these follow on as an inevitable consequence of the MLL fusion gene's activity. Aetiologically, the induction of MLL gene fusion itself is the key event and epidemiological data suggest that this may arise via transplacental genotoxic exposures during pregnancy. Finally, the probability of either MLL gene fusion or TEL-AML1 gene fusion and/or TEL deletion being induced may depend upon the background genetics of the individuals at risk (i.e. by modifying the impact of the relevant exposures). Recent molecular epidemiological data has implicated genes involved in carcinogen metabolism for infant ALL with MLL fusions and certain HLA DP/PQ alleles for cALL.

References
Molecular diagnosis and monitoring of acute lymphoblastic leukaemia: applications and limitations

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Recent chromosome abnormalities can be detected in most malignant diseases and are closely associated with particular tumour phenotypes. Clonal abnormalities can be identified in approximately 65-70% of cases of childhood acute lymphoblastic leukaemia (ALL) and in 60-85% of adult ALL. In most instances the genes disrupted by these abnormalities have been identified, thus providing important insights into disease pathogenesis and normal cellular physiology. Molecular assays have been developed and a more accurate diagnosis of disease subtypes is currently available for the translocations most frequently occurring in ALL as well as in acute myeloblastic leukaemia (AML).

Molecular analysis and conventional cytogenetics: lessons from the t(12;21) translocation

Until recently, the translocation t(12;21) was considered to be of limited prognostic value due to its apparent rarity, being barely detectable in less than 0.05% of patients. Initial attempts to identify the t(12;21) translocation using the fluorescence in situ hybridisation technique (FISH) have indicated that its prevalence is largely underestimated. The ETV6 gene on chromosome 12p13 and the AM L1 gene on chromosome 21q22 have been shown to be involved in the t(12;21) translocation. In particular, the helix-loop-helix (HLH) amino terminal domain of ETV6 is fused to the runt homology domain of AM L1. A polymerase chain reaction (PCR) technique has become available for molecular detection of the translocation.

Patients with ETV6/AM L1-positive ALL are usually young (more than 75% are under 5 years old), have relatively low white blood cell (WBC) counts at diagnosis (less than 20x109/L WBC in more than 60% of the patients) and leukaemic blasts with non-hyperdiploid DNA content (in 90% of the cases). In these cases leukaemic cells express myeloid-associated antigens with high frequency. The incidence among adult ALL is very low (1-3%). The presenting features associated with the expression of ETV6/AM L1 fusion transcripts (age, WBC, phenotype) are known to be associated with a good prognosis, raising the issue of the clinical significance of this molecular lesion as an independent prognostic factor. Several investigators have reported favourable outcomes for ETV6/AM L1-positive patients in both retrospective analyses and prospective analyses with relatively short follow-up. In particular, the two studies from the Dana-Farber Cancer Institute (DFCI) and St. Jude Research Hospital demonstrated a very high relapse-free survival in ETV6/AM L1-positive patients with long follow-up and very low frequency of ETV6/AM L1-positive relapsed patients.

However, at least four analyses of patients with relapsed ALL have shown that the frequency of ETV6/AM L1 positivity at relapse is similar to that at diagnosis. Two of these studies showed that the duration of initial remissions was longer in ETV6/AM L1-positive patients. ETV6/AM L1-positive relapsed patients also had a significantly higher probability of event-free survival after relapse therapy. There are several possible explanations for these differences. First, there might be a selection bias in the analyses, because in most cases the samples were tested retrospectively, based on availability. Second, it is possible that the high incidence of ETV6/AM L1 at relapse corresponds to a therapy-related malignancy which is ETV6/AM L1-positive. However, when a paired diagnostic sample was available, the ETV6/AM L1 positivity at diagnosis was confirmed. Third, it is possible that the different incidence of ETV6/AM L1-positive patients among relapsed patients reflects differences in the efficacy of the therapy for ETV6/AM L1-positive patients in the specific trials. For instance, it is possible that ETV6/AM L1-positive patients represent a biologically distinct subset which is more effectively treated by the agents used more intensively by the DFCI group, such as L-asparaginase. Recently, we have analysed the outcome at five years of a subset of patients with good outcome treated within the closed Italian ALL-AIEOP 91 protocol. Among the 52 patients found to be positive at diagnosis for the presence of the t(12;21) translocation, 9 relapsed. Interestingly, 7 of them were treated in the randomised arm with low L-asparaginase doses; in contrast only 2 had received high doses of L-asparaginase, suggesting the L-asparaginase could play a role in the outcome of the t(12;21) positive patients. It is likely that large ongoing prospective trials will confirm the favourable outcome of patients with ETV6/AM L1.

Towards risk classification according to genetic lesions

The availability of molecular methods to detect the most prognostic relevant chromosomal translocations in childhood ALL, has suggested that genetic features of leukaemic cells are used as a tool for classifying patients risk. To date, the only subset of B-llin-
eage ALL cases with favourable prognosis has been the hyperdiploid group, and DNA index (>1.16) is currently included as a criterion to identify a subgroup of ALL patients considered for less intense chemotherapy.² Hyperdiploidy with 47 to 50 chromosomes is seen in 5% to 15% of cases of adult ALL.² Although several studies have identified a trend towards an improved outcome with hyperdiploidy, the association with a favourable outcome is less obvious than in childhood ALL. Hypodiploidy is found in 2% to 8% of ALL cases and is associated with a poorer outcome.

As previously mentioned, no definitive conclusions can be drawn on the prognostic impact of ETV6 gene rearrangements. By contrast, the t(9;22), the t(4;11) and other MLL gene rearrangements are associated with a poor prognosis in patients treated with a variety of regimens, both in adult and paediatric ALL.²,6 MLL gene rearrangements occur in most of the cases of infant leukaemia (60% to 70% of the cases)³ and may account for the dismal prognosis in this subgroup. It has been demonstrated that the rearrangement of the MLL gene confers a poor prognosis even in children over 1 year of age, in whom it occurs less frequently (30% of the cases) than in infancy. In a study of 100 patients treated in the Dutch Bone Marrow Transplantation and Leukaemia Group, a poor prognosis was associated with the presence of a rearranged MLL gene and 64% for other cases. When infants were excluded from the analysis, MLL rearrangement was still associated with a poor outcome (p=0.02), and remained so with the exclusion of the t(4;11)-positive cases, although the number of patients included were very small. Adult patients with t(4;11) ALL have generally done poorly with conventional chemotherapy, there is some evidence that intensive consolidation may benefit this group.² In the German multicentre ALL trial, a disease-free survival rate of 43% was seen at 3 years in a small group of t(4;11) patients using an intensive regimen involving early consolidation with high-dose cytarabine and mitoxantrone. Even better results have been obtained with allogeneic transplantation with a disease-free survival rate greater than 60% in this study. Confirmation in larger prospective trials is required.

The prognostic significance of the t(1;19) appears to be dependent on the intensity of the treatment used; contemporary intensive chemotherapy regimens have abolished the poor prognosis once associated, at least in children⁴, to this translocation.

Parallel to the recent research on molecular markers, investigation of cellular and clinical response to therapy has contributed significantly to the identification of sub- sets of patients with higher probabilities of being resistant to therapy or relapsing. There is an interesting recent demonstration that, even within genetically homogeneous subgroups - such as the t(9;22)-positive ALL - the pattern of early clinical response to treatment might be significantly different.⁵ Overall it is likely that the identification of discrete genetic lesions, shown to be prognostically relevant, in combination with the evaluation of early response to treatment, will help to modulate the treatment of ALL in future clinical trials. In this context, reverse-transcription-PCR (RT-PCR) of the fusion transcripts created by the t(9;22), t(4;11) and t(1;19), may be used to assess minimal residual disease (MRD) and to identify patients with different outcome within the same genetically identified subgroup.

Recent studies of MRD following intensive combination chemotherapy regimens (without transplantation) provide intriguing preliminary evidence that achievement of a BCR/ABL negative state, as detectable by PCR, may be predictive of durable remissions in a subset of adult Ph+ ALL.⁶ Along the same line, Schrappe et al. reported that, in a retrospective study on BFM and AIEOP childhood Ph+ ALL, the early response to prednisone treatment emerged as the only independent prognostic factor for survival in Cox regression analysis.⁷ More recently, we have monitored childhood ALL patients treated within the Italian ALL-AIEOP 95 protocol by the highly sensitive RT-PCR method. This monitoring revealed a great heterogeneity with respect to MRD levels, even within the Ph+ ALL responding well to prednisone. Thus, MRD evaluation in PGR Ph+ ALL patients could help to identify patients persistently negative, that can be cured only by intensive chemotherapy.

In patients with the t(4;11) translocation the sequential analysis of the MLL-AF4 hybrid transcript showed a persistently negative RT-PCR in five long-term survivors in continuous complete remission (CCR).¹² By contrast the PCR analysis resulted persistently positive in the remaining seven cases including the four cases who relapsed after achievement of clinical CR. Similar results were recently confirmed in a prospective series of 411-positive adult ALL patients (Cimino et al., unpublished observation).

In ALL patients with the t(1;19) translocation, preliminary data in small cohorts of patients have indicated a good correlation between the presence of this transcript and relapse. More recent data have not confirmed the predictive value of MRD detection of E2A-PBX1 chimeric mRNA at the end of consolidation and patient outcome.¹² The need for standardised molecular methods for the detection of the most frequent translocations occurring in leukaemia has emerged as one of the most relevant issues for clinical studies. This standardisation was the aim of the European BIOMED-1 Concerted Action “Investigation of minimal residual disease in acute leukaemia: international standardization and clinical evaluation”, involving participants from 14 laboratories in eight European countries.¹³ In this context, standardisation and quality control were studied for the nine most frequent and well-defined chromosome aberrations with fusion gene transcripts: t(1;19) with E2A-PBX1, t(4;11) with MLL-AF4, t(8;21) with AML1-ETO, t(9;22) with BCR-ABL p190 and BCR-ABL p210, t(12;21) with ETV6-AM L1, t(15;17) with PM L-RARα, inv(16) with CBFB-MYH11, and microdeletion 1p32 with S1L-TAL1. A common protocol was designed, tested and adapted, resulting in a standardised RT-PCR protocol. The collaborative efforts resulted in standardised primer sets with a minimal target sensitivity of 10⁻² for virtually all single PCR analyses, whereas the nested PCR analyses generally reached the minimal target sensitivity of 10⁻⁴. The standardised RT-PCR protocol and primer sets can now be used for molecular classification of acute leukaemia at diagnosis and for MRD detection during follow-up to evaluate treatment effectiveness.

⁵¹⁴th Congress of the European Haematology Association - Educational Book
Minimal residual disease in ALL: a new tool for risk classification?

The study of minimal residual disease (MRD) has drawn great interest in clinical oncology because of the potential of tailoring treatment and the possibility of gaining insights into the nature of a cure. Several methods have been proposed to detect MRD in leukaemias; there is a thorough review.14

In ALL rearrangements in which immunoglobulins (Ig) and T-cell receptor (TCR) genes result in unique recombination of variable (V), diversity (D) and joining (J) gene segments, the junctional regions between these gene segments can be regarded as fingerprint-like sequences due to the deletion and random insertion of nucleotides during the rearrangement process. PCR-based MRD detection via clone-specific junctional region probes, which is designed according to the junctional region sequences of the leukaemic cells at diagnosis.

Several retrospective and limited prospective studies, indicated that the detection of MRD in childhood ALL has prognostic value, although the results of these studies were not fully concordant.14 Absence of residual disease after remission induction was associated with a good prognosis. However, approximately one half of the patients are MRD positive at that time; so the level of MRD positivity was evaluated and found to correlate with the outcome. If multiple BM samples are analysed during the follow-up, a steady decrease of MRD levels to negative is associated with a favourable prognosis, whereas persistence of MRD is generally a harbinger of clinical relapse. Of course, the level of sensitivity reached by the method used, is a crucial issue to be considered in the interpretation of the data.

Recently, two independent studies provided evidence that MRD state at the end of induction treatment correlates with outcome.15,16 In order to obtain more information on how MRD can be applied to the clinical decision process, a large prospective multicentre study was performed in 240 children with ALL, treated according to national protocols of the I-BFM Study group (I-BFM-SG) in Germany, Austria, Italy and the Netherlands.12 MRD negativity at the various follow-up times was associated with low relapse rates (3-15% at 3 years), but five-fold to ten-fold higher relapse rates (39-86% at 3 years) were found in MRD-positive patients. The distinct degrees of MRD appeared to have independent prognostic value at all separate time points, especially the first two (at the end of induction treatment and before consolidation treatment). A high degree of MRD (10^-2) at both these times was associated with a three-fold higher relapse rate in comparison with the rate in patients with a low degree of MRD (10^-4). These results were further confirmed in and extended to B-cell precursor ALL classified as intermediate risk in which relapse cannot be predicted using clinical and biological features at diagnosis. Different clearances were seen in T-ALL compared to B-cell precursor ALL. All these data provided a rationale for the design of MRD-based stratification within the new common AIEOP-BFM childhood ALL treatment protocols.

The MRD-based risk group assignment in the aforementioned studies was based on the kinetics of tumour reduction, so it is clear that the level of MRD needs to be defined precisely in follow-up samples. However, current PCR methods do not allow easy and accurate MRD quantification. More recently, real-time quantitative PCR (RQ-PCR) has been used for MRD detection in ALL. In detail, junctional regions are chosen to locate patient-specific fluorescent TaqMan probes.18 The RQ-PCR technique appears to be a sensitive, reproducible and fast method for quantifying MRD, but the method is too expensive to be applied in clinical studies. An alternative approach which has been recently proposed for IgH and TCR genes, of uses TaqMan an probes designed on IgH and TCR germline regions.19 With this approach, an allele-specific oligonucleotide (ASO) primer confers specificity to the PCR assay (Figure 1). This method has been preliminarily shown to be feasible in a series of 25 paediatric patients with T-ALL enrolled within the ALL-AIEOP-95 BFM-based therapy protocols.19 In 22 cases (88%) we succeeded in obtaining a patient-specific TcRg sequence. In all the cases we were
able to design a unique optimal TaqMan probe, greatly reducing the costs of the RQ-PCR analysis.

RQ-PCR is also becoming a very useful tool for the MRD analysis in patients carrying chromosomal translocations. The standardisation and quality control of this technique is the aim of the current Europe Against Cancer Program RQ-PCR for early diagnosis of relapse risk in leukemia, involving more than 25 laboratories in several European Countries.

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References
Risk-adapted treatment for childhood acute lymphoblastic leukaemia: results from the ALL-BFM Study Group

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At diagnosis of acute lymphoblastic leukaemia (ALL), biological features such as the age of the patient, white blood cell count (WBC), organ involvement, immunophenotype, and cytogenetics have been used by almost all major study groups to stratify treatment composition and intensity. Recently, treatment intensity has also turned out to be a major prognostic factor. The BFM group addressed the impact of treatment intensity in randomised trials (ALL-BFM 81 and 83) conducted from 1981 to 1986. The importance of delayed reintensification was confirmed in the ALL-BFM 86 trial. The prognostic influence of early treatment response was prospectively evaluated in the ALL-BFM 83 trial, and later utilised in trials ALL-BFM 86 and 90 to identify the group of ALL patients at highest risk of relapsing. Various approaches to determine early in vivo response have been developed over the last 15 years mainly concentrating on the cytomorphological evaluation of blast cell clearance from the peripheral blood and the bone marrow. Only recently have more sensitive methods of minimal residual disease detection been applied systematically to evaluate the in vivo response. In parallel to recent advances in risk-adaptation of treatment some important attempts have been made to reduce the toxicity by limiting, in particular, the components with the potentially most relevant long-term toxicity such as radiotherapy. This paper summarises the information on prognostic factors in childhood ALL as derived from the ALL-BFM trials, and examines the clinical relevance with respect to future development of treatment strategies.

Patients and Methods
Between 1981 and 1995, 4,417 patients with ALL were treated in four consecutive BFM trials ALL-BFM 81, 83, 86 and 90. Patients were enrolled in up to 96 institutions in Germany, Switzerland and Austria. Therapy for patients with B-precursor or T-ALL was risk-adapted by stratifying according to the BFM risk factor (BFM RF) calculating the leukaemic cell load at diagnosis (RF = 0.2 log (B+1) + 0.06 L + 0.04 S) [B = number of leukaemic blasts/µL peripheral blood; L and S being the enlargement of liver and spleen in cm below the costal margin]. After the prognostic significance of blast cell reduction in peripheral blood had been recognised, in vivo steroid response was utilised for treatment stratification in subsequent trials (ALL-BFM 86 and 90): patients with inadequate response to the prednisone prephase qualified for intensified therapy in the high risk group. Patients with B-ALL were treated with a different protocol. The major components of therapy have already been published in detail.

Results
Clinical and biological parameters at diagnosis
White blood cell count (WBC), age, gender, cytogenetic and immunophenotypic subtypes are the major factors determining the risk of relapse. To determine the risk of relapse it is useful to rely on parameters which are always available and not subject to methodological variations. Age and WBC are ideal parameters, as these factors are always available. Therefore, standard (SR) and high risk (HR) patients have been defined by age/WBC subsets, e.g. in the classification of the National Cancer Institute: SR, age 1-9 years, and WBC <50,000/µL; HR, age ≥ 10 years or WBC ≥50,000/µL. The BFM group prepared results accordingly, thus demonstrating a 6-year event free survival (EFS) of 86±1% for SR patients (n=1395), and of 64±2% for HR patients (n=724) in the ALL-BFM 90 trial. Infants who were not included in the NCI definition were also analysed: their 6-year EFS was 50±7%. With regards to the risk groups defined in the ALL-BFM 90 trial, 6-year EFS was 85±2%, 82±1, and 34±3% for SR (n=636), MR (n=1299), and HR (n=243) patients, respectively. Molecular screening for fusion genes and cytogenetics at diagnosis are useful methods for identifying some high risk patients, but the majority of patients at risk will not be recognised.

Response evaluation
Early response to prednisone
Early therapy response to 7 days of prednisone (PRED) and one intra-thecal (IT) injection of MTX was prospectively evaluated in the ALL-BFM 83 trial. Analysis of the results identified a novel poor prognosis marker that was more predictive of relapse than any other marker used thus far: 8% of the patients formed a small poor risk group that was characterised by the presence of >1,000 leukaemic blasts per µL peripheral blood after the first week of prednisone (PRED-PR). After a median observation time in trial ALL-BFM 83 of 10 years the probability of event-free survival (pEFS) for patients with PRED-PR is 39% compared to 66% in patients with adequate response to prednisone. No other mathematical model nor any other single factor could describe a
group of patients that was as large, and had a prognosis of less than 50% pEFS. Reflecting the reliability of this method, 8-10% of the patients have always been identified as PRED poor responders in all three BFM trials since 1983. In addition, some characteristics have commonly been observed in these trials\(^5\)\(^8\) that indicate that this parameter is associated with some well known poor risk features (Table 1). No treatment variation or intensification that was applied in trials ALL-BFM 86 and 90 for patients with PRED-PR significantly altered the outcome of this group.\(^1\) A significant improvement for patients with PRED poor response was, however, demonstrated in the ALL-BFM as trial (unpublished data). Other study groups also utilised the prognostic significance of blast cell reduction in peripheral blood (PB) or bone marrow (BM)\(^2\)\(^9\)\(^11\)\(^20\).

Response in the Bone Marrow

Since 1990, BM on day 15 of therapy has been evaluated routinely in the central laboratory of the ALL-BFM trial. Evaluation of the BM slides was associated with more technical problems than the evaluation of blood smears, and low cellularity often prevented conclusive results. In addition, the specificity of the BM evaluation was limited: the same number of relapses was observed among patients with M 1, or M 2, or M 3 marrows on day 15. Nevertheless, an M 3 marrow – found in 13% of the patients – was an indicator of high relapse risk, as 6-year EFS was only 44±5%. Among patients who were good-responders to PRED, an M 3 marrow was demonstrated in 7.3% of the patients, with an EFS of 54±7%. Failure to achieve remission as shown by a M2 or M3 BM at control time (in BFM, day 33 of induction), was the most adverse prognostic factor in the ALL-BFM 90 trial, since 6-year EFS was only 11±5% for such patients. This poor risk subset comprises, however, only 2.5% of all patients.

Response as assessed by evaluation of minimal residual disease

Highly sensitive, clonospecific detection of minimal residual disease (MRD) during treatment of ALL has been shown to be a new tool for prognostic evaluation of patients with childhood ALL.\(^2\)\(^12\)\(^13\) A collaborative study of four laboratories within the International BFM Study Group identified three distinct risk groups on the basis of semiquantitative MRD determination at 5 and 12 weeks of therapy.\(^13\)

Key elements of treatment

Reinduction therapy

In the ALL-BFM 83 trial, 126 patients with standard-low risk (SR-L) ALL (BFM-RF <0.8, no initial CNS disease: WBC <10,000 in 95% only approximately 25% of the patients being older than 10 years) were randomised to receive or not to receive reinduction therapy with Protocol III.\(^7\) Reinduction therapy was scheduled to start two weeks after the end of consolidation therapy, that is 23 weeks after diagnosis (arm SR-L/2). Patients randomised not to receive reinduction therapy (SR-L/1) were started on maintenance therapy with oral 6-MP and MTX two weeks after consolidation.\(^13\)\(^14\) Omitting reinduction increased the relapse rate 2.5-times from 17% to 41% resulting in an EFS of 59±6% for patients treated without reinduction, and 83±5% for patients who had received reinduction. This result was reproduced in the initial cohort of standard risk patients in the subsequent ALL-BFM 86 trial in which even the introduction of high-dose methotrexate in consolidation could not prevent this high rate of relapses. The majority of relapses were systemic recurrences. Since 1988, however, intensive reinduction therapy is a key element of BFM therapy also for all standard risk patients.

Maintenance therapy: treatment duration

Irrespective of their risk features 764 patients were randomised in trials ALL-BFM 81 and ALL-BFM 83 to 18 or 24 months of total therapy duration. All other patients (n=345) were chosen for one or the other treatment arm (145 patients for 18 months, 200 patients for 24 months). All events prior to the 18th month of therapy were censored from the analysis. Thus, the probability for event-free interval (pEFI) is 79% after a medium observation time of 10 years (range 8-12 yrs) for patients randomised for 24 month of treatment, but only 71% for patients randomised for 18 months (p=0.0097). The majority of late relapses occurred in patients with B-precursor ALL, thus, extended maintenance therapy could prevent a significant portion of relapses in that subgroup: pEFI is 72% for 24 months, vs. 62% for 18 months (p=0.01). In contrast, patients with T-ALL rarely experienced a relapse more than 18 months after diagnosis. Comparing the randomised groups within the various risk groups defined by the cell load at diagnosis (BFM-RF), the effect of prolonged maintenance therapy could be demonstrated for all risk groups, but only in the HR group was the extended therapy significantly (p=0.02) better than that of 18 months (pEFI 78% vs. 68%). The long-term observation reveals that patients defined as medium or high risk ALL by a BFM-RF ≥ 1.2 who entered this randomisation fared just as well as those with standard/intermediate risk features (defined in this trial by a BFM-RF <1.2):

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>PRED-PR (%)</th>
<th>PRED-GR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boys</td>
<td>67</td>
<td>55</td>
</tr>
<tr>
<td>WBC &gt;50,000 per µL</td>
<td>62</td>
<td>16</td>
</tr>
<tr>
<td>T-ALL</td>
<td>45</td>
<td>10</td>
</tr>
<tr>
<td>No CR after 5-wk induction</td>
<td>16</td>
<td>0.9</td>
</tr>
<tr>
<td>EFS ±SE*</td>
<td>48±5%</td>
<td>78±2%</td>
</tr>
</tbody>
</table>

(PRED-PR = prednisone poor response, PRED-GR = prednisone good response, for definitions see text. *Result from trial ALL-BFM 86 (6-yr EFS) (ref. #5).
pEFS in M R/H R patients is 71/80%, and in SR patients 71/79% for 18/24 months of treatment, respectively.

Consolidation phase

Since the ALL-BFM 86 trial, high-dose methotrexate (HD-MTX), i.e. 5g/m² given in 24h (four cycles) was introduced into the consolidation phase, and replaced intermediate dose MTX (0.5g/m²). The aim was to control extramedullary, in particular CNS, recurrences in order allow preventive radiotherapy to be eliminated or reduced. CNS relapses were controlled by this approach very effectively, since in the ALL-BFM 90 trial only 1.0% isolated, and 1.9% combined CNS relapses have been observed while preventive cranial radiotherapy (12 Gy) was applied only in medium and high risk patients.1

The major advantage of HD-MTX was demonstrated for T-ALL patients with adequate response to prednisone: EFS improved from 58±7% (trial ALL-BFM 83) to 82±4% and 78±3% in trials BFM 86 and 90, respectively (p=0.002). In T-cell lymphoblastic lymphoma the introduction of HD-MTX was certainly an important contributing factor in improving the outcome to 90% EFS.24 Due to the high efficacy of CNS-protection, HD-MTX also allowed us to omit IT MTX in maintenance therapy in contrast to the procedures of many ALL study groups.

Preventive cranial radiotherapy (pCRT)

Since the ALL-BFM 81 trial, the BFM group has systematically tried to reduce or eliminate preventive cranial radiotherapy in children with ALL. The first attempt to replace preventive cranial radiotherapy (pCRT) by intermediate dose MTX failed in the ALL-BFM 81 trial.24 Subsequently, it was demonstrated that 12 Gy was as effective as 18 Gy for pCRT.1 In the current trial, ALL-BFM 95, 12 Gy is only applied in T-cell ALL and HR patients.

Discussion

The prognostic significance of leukaemia specific biological factors is undoubted. Initial diagnostic procedures therefore have to aim at obtaining as much information as possible and using the combination of immunological, cytogenetic, and molecular genetic findings for optimal risk adjustment of therapy. There are, however technical and logistical limitations to retrieving all this information from all patients, especially in large multicentre trials. With the exception of rather rare, well-defined cytogenetic abnormalities, easily available factors such as WBC, age and sex have also successfully been used for risk assessment.6,25,26 Analysis of early therapy response has provided an additional instrument to detect increased risk of failure in ALL therapy.8,27 It was also shown that this rather simple and universally applicable tool was even effective in the well-characterised subset of ALL patients with t(9;22).28 All risk parameters must be investigated for their overall prognostic relevance as demonstrated in Table 2. This means that identification of a rare biological factor which is correlated with poor prognosis might not be very relevant for the total cohort, as group outcome will not change much even when patients with that parameter can be treated more adequately. On the other hand, if the unfavourable parameter is associated with a large number of recurrences, new and more effective therapy might improve overall outcome substantially. In addition, if a parameter such as high persistent level of MRD is highly specific and is correlated with a dismal prognosis, it is justified, and even mandatory, to develop experimental therapies. Such a rational approach in utilising the specificity of response parameters (including MRD evaluation) is being initiated now by a large multicentre trial of the AIEOP and BFM ALL Study Groups in Germany, Austria and Italy. This study will still include the successful principle of

Table 2. Clinical and biological parameters at diagnosis: prognostic relevance (Data from trial ALL-BFM 90). Overall prognostic relevance: qualitative assessment of potential improvement in outcome (EFS) for the total group, if the outcome in the negatively selected subset (worst category) could be improved significantly. This estimate depends on group size, EFS, and the proportion of events included in that subset.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Best category</th>
<th>% of group</th>
<th>6y-pEFS (SE) %</th>
<th>% of all events incl.</th>
<th>Worst category</th>
<th>% of group</th>
<th>6y-pEFS (SE) %</th>
<th>% of all events incl.</th>
<th>Overall prognostic relevance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>1-5y</td>
<td>60.4</td>
<td>83 (1)</td>
<td>44</td>
<td>&lt;1y</td>
<td>2.7</td>
<td>50 (7)</td>
<td>6</td>
<td>+</td>
</tr>
<tr>
<td>Gender</td>
<td>female</td>
<td>42.1</td>
<td>82 (1)</td>
<td>34</td>
<td>male</td>
<td>57.9</td>
<td>75 (1)</td>
<td>66</td>
<td>++</td>
</tr>
<tr>
<td>WBC/µL</td>
<td>&lt;10,000</td>
<td>45.9</td>
<td>85 (1)</td>
<td>30</td>
<td>&gt;100,000</td>
<td>12.4</td>
<td>52 (3)</td>
<td>27</td>
<td>++</td>
</tr>
<tr>
<td>CNS involvement</td>
<td>no</td>
<td>92.7</td>
<td>79 (1)</td>
<td>86</td>
<td>yes</td>
<td>2.5</td>
<td>48 (7)</td>
<td>6</td>
<td>+</td>
</tr>
<tr>
<td>NCI subgroups</td>
<td>SR</td>
<td>65.8</td>
<td>86 (1)</td>
<td>42</td>
<td>HR</td>
<td>34.2</td>
<td>64 (2)</td>
<td>57</td>
<td>++</td>
</tr>
<tr>
<td>Immunosubtype</td>
<td>common</td>
<td>64.6</td>
<td>82 (1)</td>
<td>49</td>
<td>pro-B</td>
<td>4.9</td>
<td>54 (5)</td>
<td>10</td>
<td>+</td>
</tr>
<tr>
<td>PRED response</td>
<td>PRED-GR</td>
<td>90.5</td>
<td>82 (1)</td>
<td>71</td>
<td>PRED-PR</td>
<td>9.5</td>
<td>34 (3)</td>
<td>29</td>
<td>++</td>
</tr>
<tr>
<td>MRD</td>
<td>MRD neg. at 5 and 12 w.</td>
<td>42.6</td>
<td>98 (2) *</td>
<td>3</td>
<td>MRD ≥10-3 at 12 w.</td>
<td>15</td>
<td>16 (8) *</td>
<td>53</td>
<td>+++</td>
</tr>
</tbody>
</table>

* Relapse-free survival at 6 years; MRD: minimal residual disease.
"over-treatment" but it will attempt to limit the "over-treatment" to a large, well-defined subset of patients to avoid the late effects which have been and still are being found in children treated for ALL.

Treatment intensity is well known to have a major impact on event-free survival in leukaemia.4-6,29-32 Two randomised trials of the ALL-BFM group demonstrated that rather late intensification of treatment is effective in preventing some subsequent relapses. First, extension of the maintenance therapy from 18 to 24 months can decrease the rate of relapses for both sexes by approximately 7%. This advantage is independent of the initial BFM risk factor but limited to patients with B-precursor ALL, and not impaired by any increase in toxic events. Secondly, late intensification is indeed important in low-risk ALL patients which limits the possibilities for eliminating multidrug regimens.12,25 More recently, we demonstrated in a large number of patients that increased dose intensity in induction can reduce the relapse rate in intermediate risk patients despite reduction of anthracyclines and pCRT.3

It is quite easy to describe a low-risk group, and a high-risk group in childhood ALL, but the characteristics of the intermediate or medium risk group that predict treatment failure are more difficult to define. The majority of relapses in an unselected group of patients will occur in individuals who were not previously identified as having the special risk features found in patients in the high risk group: in the ALL-BFM 86 trial, 135 (58%) of 233 relapses (of 998 patients enrolled) occurred in medium risk patients whereas 60 (25%) occurred in standard risk and 38 (16%) in high risk patients. Identification of new genetic markers of aberrant drug metabolism as well as the functional understanding of resistance to chemotherapy are interesting approaches to preventing the large number of "unpredictable" relapses by developing a better understanding of the "host".

References


5th Congress of the European Haematology Association - Educational Book


Prions and cells of the immune system

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Transmissible spongiform encephalopathies (TSEs) comprise a spectrum of diseases in animals and man. In 1985 a novel TSE was identified in domestic cattle in the United Kingdom. Bovine spongiform encephalopathy (BSE) is thought to have been transmitted by feeding cattle ruminant-derived meat and bone meal and led to an epidemic of over 175,000 clinical cases in the UK by November 1998. Mathematical calculations suggest that around 750,000 asymptomatic but infected cattle are likely to have been slaughtered and entered the human food-chain in the 1980s and early 1990s. BSE is known to have crossed several species barriers giving rise to TSE in domestic and exotic cats, exotic ungulates and a variety of other species. In July 1998 concern over the possible transmission of BSE to man led the UK Government to ban the feeding of ruminant dried meat and bone meal to cattle and in November 1998 human consumption of specified bovine offals was banned.

In 1996 a new form of human transmissible spongiform encephalopathy, termed variant Creutzfeldt-Jakob disease (vCJD) was described by the UK Creutzfeldt-Jakob Disease Surveillance Unit in Edinburgh. vCJD differs from sporadic or classical Creutzfeldt-Jakob disease (spCJD) in that patients present at a younger age (median of 29 years) with behavioural changes, dysaesthesia and ataxia, developing progressive dementia and death after a period of 7.5 to 22.5 months. The neuropathological features include spongiform change, neuronal loss and astrogliosis, along with remarkably florid prion plaques and deposits. The clinical, neuropathological and epidemiological features of vCJD suggest a relationship with BSE and more recent experimental data has strongly supported this analysis. Collinge et al have demonstrated by Western blot that the proteinase K resistant PrP glycoform pattern seen in vCJD is similar to that of BSE in cattle and other animals and dissimilar to those seen in spCJD. Bruce et al have shown that the pattern of incubation period and neuropathological targeting of vCJD in a TSE strain typing panel of inbred strains of experimental mice is similar to that of BSE and differs from that of spCJD. The evidence that vCJD and BSE are the same strain of TSE is now compelling. Forty-eight cases of vCJD have currently been identified in the UK with a further two in France and one in Ireland. Calculating the number of people currently incubating vCJD who may eventually develop clinical disease is fraught with difficulty. The dose of BSE-infected material required to transmit across the species barrier to man is unknown. Mathematical projections based on the current number of cases of vCJD and the likely range of median incubation periods, estimate that there may be between 80 and 80,000 individuals in the United Kingdom currently incubating the disease. Most authorities agree that it is too early to predict with any degree of certainty whether the UK is facing a relatively small number of cases or a large epidemic over the next 30-40 years. A primary concern therefore is that because vCJD represents a different strain of disease from spCJD, it may behave differently in terms of the distribution of peripheral infectivity and ease of transmission by blood components and products. The second issue is that the prevalence of pre-clinical infection with vCJD may be several orders of magnitude greater amongst UK donors (estimated prevalence 1:106 to 1:1,000) than that of other forms of CJD (prevalence around 1:106).

The pathophysiology of transmissible spongiform encephalopathies

Prion protein (PrP) is a 30-35 kd glycoprotein with 2 N-linked glycosylation sites. The predominant form of the protein is linked to the cell membrane by a glycosyl phosphatidyl inositol (GPI) anchor, though transmembrane and soluble forms have also been described. The secondary structure of normal prion protein (PrPC) consists predominantly of alpha helices with a single beta-pleated sheet within the membrane proximal portion of the protein and an unstructured membrane distal portion. The function of PrPC remains uncertain though it is known to act as a copper metalloprotein, has been shown to bind to a laminin receptor precursor protein and more recently to a proteoglycan.

TSE development is associated with conformational change in the secondary structure of PrP (termed PrPSc), and with an increase in the proportion of beta-pleated sheets mainly at the expense of the unstructured membrane-distal part of the molecule. This change in secondary structure renders the molecule resistant to biological and physicochemical degradation with a resulting abnormal accumulation of PrP as amyloid plaques. The mechanism by which PrPSc is converted to PrP and the causal relationship of this structural change to infectivity remain uncertain. It is proposed that the presence of PrP leads to conversion of PrP to the abnormal conformer either by a biological process of homod-
imerisation or by a physio-chemical process of nuclear crystallisation. Whatever the exact mechanism, the presence of PrP Sc is clearly a prerequisite for TSE infectivity, since PrP null mice are resistant to TSE transmission. Several groups including our own have studied the distribution of PrP in the peripheral haematologic and immune systems in man. Flow cytometry studies show that PrP is present on mononuclear cells and platelets. More accurate quantitative studies using dissociation enhanced lanthanide fluorescence immunoassay (DELFIA) have demonstrated around 100 ng/mL of PrP in peripheral blood, 95% of which is associated with platelets or plasma and the remainder predominantly with mononuclear leukocytes. The substrate required for TSE infectivity is therefore widely distributed in the peripheral blood of man.

The pathophysiology of peripheral transmission

The majority of cases of CJD are of a sporadic or familial nature and therefore arise within the central nervous system. However, oral or parenteral transmission can occur, and these cases are particularly informative with regard to the likely pathophysiology of vCJD. Kuru was described in the Fore people of Papua New Guinea by Cajal in the late 1950s; it is a human TSE transmitted by ingestion of human brain during funeral rites. CJD has been transmitted by iatrogenic administration of pituitary growth hormone and gonadotrophin derived from pooled human cadaveric pituitary. These cases showed a similar pattern of clinical disease to that seen in vCJD and together point to a median incubation period of around 15 years with a range from 5 years to up to 40 years or beyond. The route by which peripherally transmitted TSE spreads to the central nervous system is a key issue, with most of the experimental data pointing to a central role for the immune system in mediating this process. Infectivity and/or PrP Sc can be detected in the peripheral lymphoid tissue of scrapie-infected sheep and rodents from shortly after inoculation, well before the development of central nervous system disease. Immunopotentiation has been shown to increase the efficiency of peripheral scrapie transmission, and immunosuppression to decrease such transmission. Splenectomy also significantly prolongs the incubation period of the disease. Mice with severe combined immunodeficiency are resistant to peripheral but not cerebral transmission of TSE and sensitivity to peripheral transmission is regained following allogeneic bone marrow transplantation. Experiments in PrP knock-out mice and those selectively deficient in some cellular components of their immune system, have suggested that B-lymphocytes and follicular dendritic cells play a key role in peripheral disease.

In man, abnormal accumulations of PrP do not occur in the peripheral lymphoid system of patients with sporadic CJD, but have been demonstrated in follicular dendritic cells in the peripheral lymphoid tissue of patients with vCJD. It therefore appears that peripheral B-lymphocytes and follicular dendritic cells are likely to be involved from an early stage following the peripheral transmission of vCJD in man, although it is possible that other cells and tissues may also carry infectivity, particularly given the aforementioned distribution of PrP in peripheral blood of normal individuals.

Evidence that TSEs may be transmissible by blood transfusion

Several studies have claimed to demonstrate experimental transmission of spCJD from whole blood or Buffy coat of clinically affected individuals by intracerebral inoculation into experimental rodents. These data have not been widely replicated, and similar transmission studies to primates have not proved successful. No such transmissions have yet been demonstrated from the peripheral blood of individuals with vCJD but the number of completed experiments carried out is currently small. Approximately 15% of patients with spCJD will have previously acted as blood donors and a significant number of patients must therefore have received blood components or plasma products from donors who later went on to develop disease. However, there are only a handful of clinical case reports of associations between spCJD and prior blood component usage. The majority of epidemiological case control, look-back and surveillance studies have shown no evidence of increased risk of spCJD in those who have received blood components or plasma products. Unfortunately these data cannot be taken as reassurance that vCJD will behave similarly, given previous comments that it represents a different strain of the disease with a probable longer incubation period, shows greater involvement of peripheral lymphoid tissue, and that the number of donors currently incubating the disease may be several orders of magnitude higher than that of donors incubating other forms of CJD. Key issues such as the potential presence, titre, distribution and timing of infectivity in the peripheral blood and lymphoid tissue of patients with vCJD and the titre required to transmit these by intravenous administration are currently unresolved. The level of risk faced by recipients of blood components is currently unknown. In the face of this uncertainty, a balance needs to be struck between applying precautionary principles in risk reduction and avoiding a critical adverse impact on the blood supply.

References

Novel platelets and substitutes

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Allogeneic platelets for transfusion to bleeding thrombocytopenic patients have been available for over 3 decades. The most widely used allogeneic platelet preparation available is one that is stored in the liquid state at 22°C. At present, in most jurisdictions, such liquid-stored platelets do not undergo any sterility procedures. Thus, despite many advances in their preparation (see below), allogeneic platelet products continue to be associated with risks to recipients. These include febrile non-haemolytic transfusion reactions (FNHTR); transmission of viral, bacterial, and protozoal infections; alloimmunisation resulting in refractoriness; transfusion-associated immunomodulation; and graft-versus-host disease. An additional shortcoming of the current preparation of liquid-stored allogeneic platelets is their short supply, which is related in part to their mandated short shelf-life of 5 days.

At present, the transmission of most infections by allogeneic platelet transfusions is minimized by: (a) exclusion of high risk individuals from the donor pool; and (b) serologic testing of all blood donors. To reduce the risk of transfusion-transmitted viral infections (such as HIV and HCV) further, DNA and RNA amplification techniques have recently been introduced to screen all blood donors.

There has been recognition in recent years that passenger allogeneic leukocytes contribute to many of the undesirable biological and clinical effects associated with platelet transfusions. Steps have therefore, been taken in many countries to reduce the numbers of leukocytes in allogeneic platelet preparations. This is usually done by leukocyte filtration prior to their storage. Thus, a considerable number of advances have been made in the preparation of allogeneic human platelets including: (a) the development of more effective storage containers; (b) the ability to produce single donor platelets by apheresis; and (c) the advent of effective prestorage leukoreduction techniques. These last have been used to prevent some of the biological side effects shown to be associated with the presence of passenger allogeneic leukocytes (i.e. alloimmunisation, viral transmission, and FNHTR).

Despite these improvements, adverse effects in transfusion recipients continue to occur. Much effort, therefore, has been expended in the development of approaches to produce safer and haemostatically active novel platelet products and substitutes that might be effective in the treatment of the bleeding thrombocytopenic patient. It has been envisioned that such products would have a longer shelf-life than the currently available liquid-stored allogeneic platelets (random donor and apheresis).

Many approaches have been explored experimentally to produce haemostatically active platelet products that can be stored for a long time. These include platelet storage in the frozen state, storage in the liquid state in the cold, use of rehydrated lyophilized platelets; the use of platelet membrane microparticles, and photochemical treatment capable of pathogen (viruses, bacteria, and protozoa) inactivation. In addition to products whose primary source is human platelets, investigators have explored various approaches to make artificial products that are capable of in vivo haemostasis. These include red blood cells bearing fibrinogen or RGD peptides covalently linked to their surfaces, synthetic phospholipids and/or liposomes bearing various haemostatically active agents on their surfaces, and fibrinogen coated albumin microcapsules.

The development of novel products and platelet substitutes is an increasing technological endeavour based on evolving scientific knowledge. The outlook for the development of haemostatically effective products that might one day replace the current form of liquid-stored platelets is very promising. However, many problems remain. A major one is the unavailability of an agreed methodology for assessing in vitro or in vivo haemostatic function of such alternative products. Moreover, as these various products may be used in different clinical indications, clear-cut criteria must be available to evaluate both their haemostatic activities and their mechanisms of action.

The various putative novel platelet products and substitutes (summarised in Table 1) have utilised different strategies to achieve primary haemostasis. In general, they have been designed either to replace platelets with modified or artificial platelets, to augment the function of existing platelets, and/or to provide a pro-coagulant surface for achieving primary haemostasis in the thrombocytopenic patient.

With few exceptions, any putative novel platelet product or substitute should function haemostatically in vivo. While the primary focus of research has been to develop products that are haemostatically effective, one must acknowledge that platelets have...
Cold-stored liquid platelets

Although the storage of allogeneic platelets in the cold retards the growth of bacteria, when present, liquid-suspended platelets stored at 4°C, have been shown to have short in vivo survival and not to be effective haemostatically. Such platelets are said to have sustained the cold storage lesion. The pathophysiological events underlying the cold storage lesion remain poorly understood. Nonetheless, a number of new strategies for liquid cold storage have evolved recently in an attempt to store platelets at 4°C and preserve platelet morphology and function. These include: the inhibition of cytoskeleton actin assembly; the use of platelet second messenger inhibitors; and the use of antifreeze glycoproteins (AFGP). These last are proteins isolated from several species of fish that have adapted to survive in the extreme conditions occurring in the polar regions of the planet. The use of AFGPs has been shown to reduce the platelet activation that has been associated with the storage of human platelets in the cold. Nevertheless, in preliminary studies, cold liquid-stored platelets, even when stored in the presence of AFGP, have been shown not to have in vivo haemostatic activity. (Blaichman MA, unpublished observations).

Photochemically treated platelets

Preparations of conventional (22°C) liquid-stored platelets treated with a combination of a photochemical agent, such as a psoralen, and ultraviolet light to inactivate any bacteria and viruses that may be present are currently being studied in phase 3 clinical trials. The available data have recently been reviewed elsewhere in some detail. Such photochemically treated platelet preparations have been shown to retain their in vivo haemostatic activity in various experimental animal models. It is very likely that preparations of such microbiologically inactivated platelets will come into clinical use within the next two to three years.

Platelet-derived microparticles

Platelet microparticles are microvesicles of platelet membranes that were first described over thirty years ago. Such microparticles form spontaneously during the liquid storage of platelets and can be found in platelet concentrates, fresh-frozen plasma, and cryoprecipitate. Platelet microparticles, like intact platelets, possess procoagulant activity; adhere to the vascular subendothelium; and enhance platelet adhesion. Because microparticles possess haemostatic properties similar to those of intact platelets, it appeared logical for investigators to examine their potential as a putative platelet substitute.

A microparticulate human platelet preparation was recently developed by Cypress Bioscience of San Diego, California, USA. This company’s infusible platelet membranes (IPM) are prepared from outdated platelet concentrates. These IPM, which consist of spherical vesicles with a diameter of approximately 0.6 µm, have been shown to contain various procoagulant phospholipids. IPM have been shown, in vivo, to shorten the prolonged ear bleeding time in thrombocytopenic rabbits for at least six hours post-infusion. However, by 24 hours the haemostatic activity was no longer detectable. Toxicity studies in experimental animals have not demonstrated pathological thrombogenicity or potentiation of disseminated intravascular coagulation in endotoxin-treated rabbits. IPM have been successfully infused into normal human volunteers in phase 1 clinical trials. Phase 2 trials have been con-
ducted in bleeding refractory thrombocytopenic patients and the results have provided an indication of improvement or cessation of bleeding in some patients.\textsuperscript{1,3,4} Phase 3 clinical trials appear imminent.

Lympholised platelets

Studies with rehydrated lympholised platelets were first carried out in the 1950s. However, studies in experimental animals with the then available preparations failed to show any haemostatic efficacy in vivo. Recently, a contemporary lympholised platelet preparation has become available and the pre-clinical results have been very encouraging.\textsuperscript{11} This contemporary preparation uses washed platelets treated with 1.8\% paraformaldehyde, frozen in 5\% albumin, and then lympholised. Rehydrated lympholised platelets are structurally similar, under electron microscopy, to fresh platelets and have been shown to bear many platelet glycoproteins, albeit at decreased concentration. The membranes of reconstituted, lympholised platelets appear capable of supporting thrombin generation and increasing fibrin deposition on exposed subendothelium in vascular perfusion models.\textsuperscript{12} In vivo, rehydrated lympholised platelets have been evaluated in dogs, rats and rabbits and in all three experimental animals have been shown to be haemostatically effective by shortening the bleeding times of thrombocytopenic animals. The duration of the haemostatic activity of these platelets in vivo seems to be short (approximately 4-6 hours). Rehydrated lympholised rabbit platelets have been shown to have a T1/2 in thrombocytopenic rabbits of approximately four hours (Blajchman MA, unpublished observations).

Platelet substitutes, not platelet-derived

Red cells with surface-bound fibrinogen, or RGD peptides

Fibrinogen is the chief ligand that cross-links stimulated platelets to result in the formation of platelet aggregates. This occurs when one of the RGD (arginine-glycine-aspartic acid) sequences within fibrinogen binds to an integrin on platelet GPIIbIIIa. Since fibrinogen possesses more than one RGD sequence, stimulated platelets can be bridged by fibrinogen to form platelet aggregates. Over the years, many in vitro experiments have shown that red cells and beads bearing fibrinogen, or RGD sequences, can cause platelets to aggregate. However, in most instances, in vivo experiments with such material have not been shown to be associated with haemostatic efficacy.\textsuperscript{1,3,4}

Fibrinogen-coated albumin microcapsules/ microspheres

The observation that either inert beads or formaldehyde-fixed platelets bearing fibrinogen on their surfaces could enhance platelet aggregation set the stage for the production of fibrinogen-coated albumin microcapsules (FAM) for potential use as putative haemostatic agents in thrombocytopenic patients. Two different FAM preparations are currently undergoing pre-clinical testing. Both preparations have human fibrinogen immobilised on microcapsules, or microspheres, composed of human albumin. Both FAM preparations have been evaluated in thrombocytopenic animal models and both have been shown to be haemostatically effective in vivo.\textsuperscript{13-15}

Liposome-based haemostatic agents

Two different approaches have been taken to develop a liposome-based platelet substitute. The first involves the use of liposomes bearing platelet membrane glycoproteins to construct an artificial platelet, while the second is based on the intravenous infusion of procoagulant liposomes, together with activated factor X (FXa). Both approaches have been shown to be haemostatically effective in vitro and in some experimental animal studies. In vivo, the use of procoagulant liposomes together with FXa has been shown to be effective haemostatically in haemophiliac dogs, however, their use was associated with unacceptable toxicity.\textsuperscript{4}

Conclusions

The development of novel putative platelet products and substitutes is an increasing scientific and technological endeavour. The current outlook for the development of haemostatically effective products that might replace or supplement the current standard 22\°C liquid-stored allogeneic platelets is very promising, despite many remaining problems.\textsuperscript{2,4} One major problem is the lack of a readily available methodology for assessing, either in vitro or in vivo, the haemostatic function of such alternative products. Moreover, as these products may be used in various different clinical situations, clear-cut criteria must be available to evaluate both their haemostatic efficacy and their mechanism of action.

With advances in technology, improved understanding of haemostasis, and the evolving field of platelet replacement therapy, there is every reason to believe that novel platelet products and substitutes will continue to become more effective in vivo and safer to use. Moreover, the insights derived from both basic sciences and clinical studies will allow better understanding of the complexities of haemostasis which in turn will lead to the design of ever more effective and safer novel platelet products and substitutes for use in patients at increased risk of bleeding due to thrombocytopenia.\textsuperscript{2}

References


Autologous blood donation

Fifteen years ago, fewer than 5% of eligible patients scheduled for elective surgery chose to pre-donate autologous blood. When public awareness of transfusion-transmitted HIV became widespread, however, several states passed legislation requiring that whenever it was reasonably likely that transfusion would be needed, the patient should be informed of all of the options and alternatives to allogeneic transfusion. Subsequently, pre-operative autologous donation increased substantially, with up to 50 to 75% of patients choosing this option prior to certain elective surgical procedures. In 1992, autologous blood units accounted for one in every twelve blood units collected and represented 5% of all blood transfused in the United States (Table 1).

Half to two-thirds of autologous blood that is collected nationally is discarded. Reasons for the over-collection may include the unnecessary collection of autologous blood for surgical procedures that do not usually require blood, such as transurethral resection of the prostate, vaginal hysterectomy, or normal vaginal delivery for childbirth. M oreover, even for surgical procedures in which crossmatched blood is indicated, pre-operative autologous donation is targeted to cover the transfusion needs for a range (up to 90%) of patients who might receive blood, which results in the routine collection of more blood than is needed (on average) for perisurgical transfusions. Increasing pressures to decrease medical care costs, along with lack of reimbursement for preoperative autologous donation from Medicare and some private insurers, have also focused attention on the over-collection of autologous units.

In view of these factors, since 1992 the percentages of autologous blood collected and transfused nationally have declined substantially (Table 1). A recent national, multicentre audit identified current autologous blood pre-donation activity and transfusion outcomes for patients undergoing total joint replacement surgery. Forty-seven to 65 percent of patients pre-donate, on average, 1.6 to 2.1 autologous blood units before procedures ranging from unilateral knee to bilateral knee arthroplasty or hip revision. Twenty-nine to 55% of these autologous units collected are wasted. Allogeneic transfusion outcomes are detailed in Table 2. The benefit of autologous blood pre-donation can be seen to reduce the likelihood of allogeneic blood exposure by approximately two-thirds for patients who are non-anemic, and by approximately one-third in patients with baseline anaemia (Hct < 39%), when compared to patients who do not pre-donate autologous blood prior to total joint arthroplasty.

Safety considerations

The re-evaluation of autologous blood pre-donation practices is, in part, related to a substantial reduction in risks from viral transmission by allogeneic blood. Autologous blood donation and the transfusion of autologous blood are each associated with risks. In one study, one in 16,783 autologous donations was associated with an adverse reaction severe enough to require hospitalisation, which is twelve times the risk associated with community donations by healthy individuals. Ischaemic events have also been reported to occur in association with autologous blood donation. The transfusion of autologous blood has many of the same complications as transfusion of allogeneic units, including bacterial contamination, haemolysis (ABO blood group-related reactions due to errors in the administration of units), and volume overload.

Pre-operative autologous blood donation may actually be harmful to patients. Figure 1 illustrates the effect of pre-operative autologous blood donation on the pre-operative haematocrit level (before blood loss) and the final haematocrit level upon hospital discharge for a 70 kg patient in which zero, two, or four units of autologous blood were pre-deposited. In essence, pre-operative autologous donation appears to cause an enhanced risk of postoperative anemia, with an increased likelihood of transfusion and its attendant risks. Advantages and disadvantages of pre-operative autologous blood donation have been reviewed recently.

The degree of anaemia induced by pre-operative autologous blood donation is variable, even though patients are routinely prescribed iron supplementation. Some authors have reported an average decrease in haemoglobin level of one g/dL per unit (i.e., no compensatory erythropoiesis) when blood was donated prior to hysterectomy, radical prostatectomy, or colectomy. The relationship between erythropoietin, iron, and erythropoiesis has been reviewed recently. The variability of compensatory erythropoiesis appears to be independent of patient’s age or gender, but dependent on initial iron status. Given that normal individuals take many weeks to regenerate the blood lost in donation and that a lower haemoglobin level admission is associated with an in-
creased transfusion requirement, it is desirable to maximise the time between the last donation and the date of surgery.

Who should pre-donate?

Recommendations for selecting patients suitable for pre-operative autologous blood donation have been published.21,22 The number of autologous units requested usually conforms to a variation of the maximum surgical blood order schedule, which determines how many blood units should be cross-matched before surgery. This approach was designed to collect enough autologous blood so that fewer than 10% of patients who successfully donate the requested number of units would subsequently be transfused with allogeneic blood. When autologous blood is collected for procedures that seldom require transfusion, up to 90% of units collected for these procedures are wasted.6

Attempts to stratify patients into groups at high and low risk for transfusion based on the baseline level of haemoglobin and on the type of procedure, show some promise. In a Canadian study using a point score system, 80% of patients undergoing orthopaedic procedures were identified to be at low risk (less than 10%) of subsequently needing transfusion, so that autologous blood procurement from these patients would not be recommended.23,24 However, one problem with algorithms which consider the estimated blood loss and pre-operative haematocrit is that blood losses are difficult to predict, so that specific surgical procedures, even by the same surgeon, can be accompanied by a wide range of blood losses.

Erythropoietin therapy

Nevertheless, anaemia is the most important determinant for transfusion risk.4,8 One important approach to this problem is iron supplementation6 before surgery, and the availability of safer parenteral iron (gluconate, saccharate) preparations holds promise in making this alternative to blood transfusion increasingly attainable. Another approach is erythropoietin (EPO) therapy, an approved alternative to autologous blood pre-donation in the surgical setting.25 In a recent, multicentre clinical trial, erythropoietin therapy was demonstrated to be equally efficacious to autologous blood pre-donation in reducing allogeneic blood exposure in patients undergoing orthopaedic surgery.26

The safety of EPO therapy in patients undergoing non-cardiac surgery has been demonstrated by the equal distribution of adverse concomitant events between patients treated with EPO or placebo in over 1,000 patients participating in clinical trials. Thrombotic events described in early trials in patients with renal failure have not been seen related to EPO use in surgical settings.27 An unresolved question is the issue of safety of EPO therapy in cardiac surgery pa-

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Table 1. Collection and transfusion of autologous blood in the United States.

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Modified, with permission, from Goodnough et al. NEJM 1999;340:439.


<table>
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<td>Revision</td>
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Figure 1. Calculated haematocrit after pre-operative donation of zero (n), two (H), or four (u) by a patient with a blood volume of 5,000 mL, and haematocrit after surgical blood losses of 500 to 5000 mL. The haematocrit is assumed to decrease by 1% (i.e., compensatory erythropoiesis replacing two-thirds of red cells removed) between each donation. The example given is for a patient with an initial haematocrit of 45% and a peri-operative transfusion trigger haematocrit level of 25%. Such a plot illustrates that for moderate to even substantial blood loss (estimated blood losses up to 2473 mL) a patient who predonated up to four units of whole blood would be discharged with a lower haematocrit level than if the patient had not pre-donated autologous blood. If the red cell regeneration between donations was less, the differences would be further magnified. Reprinted, with permission (ref. #4).
tients and its role in this setting. In a European trial, the authors found no untoward evidence of mortality, thrombotic events or serious adverse events, nor any differences in haemostatic parameters with EPO-generated levels of haematocrit (from 42±3% to 48±3%). When compared to placebo-treated patients, EPO-treated patients had improved extractable oxygen peri-operatively that was also associated with a lower incidence of lactic acidosis. An American study also concluded that there were no differences in adverse events between EPO and placebo-treated patients and that EPO therapy was well-tolerated; however, the description of adverse events and mortalities in this report indicated that an uneven distribution of these events between the placebo and EPO-treated groups could not be ruled out to any degree of certainty.

What is the current role of EPO therapy in cardiac surgery? This approach remains a valuable tool for patients with special requirements, such as Jehovah’s Witnesses for whom blood transfusion is not an option. Future trials of EPO therapy in cardiac surgery in other countries will be helpful in providing additional information on the safety of EPO therapy in this setting. Until then, the use of EPO in patients undergoing cardiac or vascular surgery in the USA cannot be recommended. Emerging data on the use of EPO in non-cardiac procedures, such as radical prostatectomy in elderly men, may provide additional evidence that mild peri-operative elevations of haematocrit, even in patients at risk of ischaemic heart disease, are safe and well-tolerated.

The potential impact of reimbursement policies are important issues in the setting of surgical anaemias, as has been the case in medical anaemias. Costs associated with EPO therapy may be lowered by strategies that improve the dose and response relationship. The pharmacoeconomics of subcutaneous administration is superior to that of intravenous administration. One study demonstrated that four weekly injections of subcutaneous EPO (600 U/kg) was less costly but was just as effective as a daily dose of EPO (300 U/kg for 14 doses). However, these regimens remain expensive, and when unaccompanied by autologous blood procurement, are still associated with an allogeneic exposure rate of 16-25%.

The most cost-effective use of EPO may be to increase the level of haematocrit in patients who are anaemic and who are anticipated to have substantial surgical blood losses. A target pre-operative haematocrit of 45% should minimise the need for allogeneic blood transfusions, especially when accompanied by prudent transfusion practice. Patients undergoing more complex surgery with substantial blood needs may require a combination of EPO therapy and autologous blood procurement. Low dose EPO therapy coupled with acute normovolaemic haemodilution has been shown to be cost-equivalent to the collection of three autologous blood units before elective surgery.

Acute normovolaemic haemodilution

Acute normovolaemic haemodilution entails the removal of whole blood from a patient immediately prior to surgery, so that losses in red-cell volume are reduced during perioperative blood loss because of the attendant lowering of haematocrit levels pre-operatively. Moderate haemodilution to maintain a pre-operative haematocrit of 28% has been estimated to save 100 to 200 mL of red cell losses, or the equivalent of one half to one unit of blood. The aim of haemodilution is to protect patients who might have unpredictable or substantial blood losses, yet maintain peri-operative haematocrit values.

A prospective study of patients who underwent acute normovolaemic haemodilution before radical prostatectomy found that 21% of patients received allogeneic blood. This rate is similar to the rate in patients who undergo autologous blood donation before radical prostatectomy and in patients who undergo autologous blood donation before elective orthopaedic surgery. Two prospective, randomised trials comparing ANH and pre-donation in patients undergoing total knee or hip arthroplasty found both techniques to be equivalent in reducing allogeneic blood exposure.

Acute normovolaemic haemodilution has several advantages over autologous blood donation. First, the units procured by haemodilution require no testing, so that the costs are substantially lower than those of autologous blood donation. Second, since the units of blood are not removed from the operating room, the possibility of an administrative error that could lead to an ABO-incompatible blood transfusion is theoretically eliminated, as is the risk of bacterial contamination. Third, blood obtained by haemodilution does not require an additional investment of time by the patient since it is done at the time of surgery, nor does it prolong duration of surgery or anaesthesia.
Intra-operative blood recovery

Intra-operative recovery of blood involves the collection and reinfusion of autologous red cells lost by a patient during surgery. The survival of the red cells that are recovered appears to be similar to that of transfused allogeneic red cells. Relative contraindications include the potential for the aspiration of malignant cells, the presence of infection, and the presence of other contaminants such as amniotic or ascitic fluid in the operative field. Because washing does not completely remove bacteria from the recovered blood, intra-operative recovery should not be used if the operative field has gross bacterial contamination.

As with other strategies of autologous blood procurement, the relative benefits, safety and costs of intra-operative recovery of autologous blood should be carefully scrutinized. A recent prospective, randomised trial of patients who were undergoing repair of abdominal aortic aneurysms found that intra-operative recovery of blood did not result in the need for fewer blood transfusions. Four deaths related to intra-operative blood recovery were reported to the New York Department of Health from 1990 through to 1995, for an estimated rate of one per 35,000 procedures. With the use of automated cell-washing devices, the equivalent of at least two units of washed blood needs to be recovered in order for this method to be cost-effective. Intra-operative recovery of blood may be of most value not because it reduces the requirements for blood transfusion, but because it provides blood that is less costly to obtain and is more immediately available in the event of rapid blood loss.

Post-operative blood recovery

The safety and the benefit of the use of unwashed blood obtained from surgical drains after orthopaedic surgery remain in question. One large group that initially found this approach to be beneficial subsequently reported that this costly practice is of no clinical benefit. Because the blood-cell volume of the fluid collected is low, the volume of red cells reinfused is often small. Selective use of the method in situations in which large post-operative blood losses are anticipated, such as in bilateral joint-replacement surgery, would improve the efficacy for the procedure, but such blood losses are difficult to predict.

Red cell substitutes

In recent years, there has been increasing interest in the development of red-cell substitutes. Efforts have included the development of cell-free haemoglobin solutions that approximate the same oxygen-carrying and oxygen-delivery capacity of cellular haemoglobin, and the development of perfluorocarbon emulsions (as synthetic oxygen carriers). The potential advantages of such products include a prolonged shelf life, the fact that they can be stored at room temperature, universal biocompatibility (since ABO-blood-group testing is not necessary), and the fact that such products are subjected to vial inactivation procedures. The disadvantages of such products include potential interference with the results of laboratory tests, their relatively short time in circulation (24 to 48 hours), and the fact that perfluorocarbons require a forced inspiratory oxygen concentration of 100% to be effective.

The two principal uses of red-cell substitutes currently under clinical investigation are for patients with acute trauma and patients who are undergoing surgery, with or without acute normovolemic haemodilution. The rationale for the use of red-cell substitutes with haemodilution is twofold: the cellular haemoglobin collected during haemodilution would be used to replace the haemoglobin solution or other synthetic oxygen carrier as it is eliminated, and the use of red-cell substitute would permit more aggressive haemodilution with lower targeted cellular haemoglobin levels that would otherwise be tolerated. However, patients with preexisting anemia can be expected to derive only limited benefit from this approach, since there is less autologous cellular haemoglobin to begin with. Moreover, studies of some haemoglobin solutions that have been administered to anaesthetised surgical patients in clinically relevant doses have demonstrated that the ability of haemoglobin-based oxygen carriers to increase oxygen delivery is limited by their vasoactivity. This vasoactivity is thought to be a direct effect of the free haemoglobin, since free haemoglobin has either a different affinity, or proximity (or both) to nitric oxide than cellular haemoglobin.

Some of these products are in later stages of clinical development, and others have failed to pass tests of safety. Some of these would most likely be used in military and trauma settings; their role in other arenas will most likely be determined by issues related to blood inventory and costs, rather than the safety of the blood supply.

Conclusions

Increased attention to the costs of health care delivery has caused the relative benefits and costs of blood conservation to be scrutinized. The prospective identification of surgical candidates who will need transfusion and therefore will truly benefit from blood conservation must be based on patient-specific factors, such as the base-line haematocrit and the anticipated blood loss during surgery. The challenge for physicians will be to educate their patients that the decision to conserve blood should no longer be based on the safety of the blood supply, but on evidence that blood conservation is safe and of value for individual patients.

References

4. Goodnough LT, Brecher ME, Kanter M, AuBuchon JP. Medical Progress: Transfusion Medicine, Part II.
5. Renner SW, Howanitz PJ, Bachner P. Preoperative auto-
6. Goodnough LT, Saha P, Hirschins NV, Yomtovian R. Autologous blood donation in non-orthopaedic sur-
7. Yomtovian R, Kruskal MS, Barber JP. Autologous-
8. Bierbaum BE, Callaghan JJ, Galante JO, et al. An-
alysis of blood management in patients having total hip or knee arthroplasty. J Bone Surg 1999; 81A:2-
10.
10. Cohen JA, Brecher ME. Preoperative autologous blood don-
ation: benefit or detriment? A mathematical-
11. Popovsky MA, Whitaker B, Arnold NL. Severe out-
12. Goodnough LT, M onk TG. Evolving concepts in au-
tologous blood procurement. Case reports of peri-
13. Kasper SM, Ellinger J, Stachwitz P, Lynch J, Grunen-
berg R, Buzello W. All adverse events in autologous blood donors with cardiac disease are not necessar-
14. Kanter MH, van M anen D, Anders KH, Castro F, Mya WW. Chloral: Perioperative autologous blood dona-
tions before elective hysterectomy. JAMA 1996; 276: 798-801.
fusions on patients undergoing radical prostatectomy using hypotensive anesthesia. J Urology 1993; 927:3-
6.
fusion-midulated tumor recurrence: first results of ran-
17. Busch ORC, Hojo WCJ, Hoyncv van Papendrecht MAW, Marquet RI, Jeekel J. Blood transfusions and pro-
19. Goodnough LT, Price TH, Parvin CA. The endogenous erythropoietin response and the erythropoietic re-
20. Goodnough LT, Marcus RE. Erythropoiesis in patients stimu-
lated with erythropoietin: The relevance of stor-
21. Consensus conference on autologous transfusion. Fi-
22. British Committee for Standards in Hematology. Preoperative autologous blood transfusion task force: guidelines for autolog-
23. Larrocque BJ, Gilbert K, Brien WF. A point score system for predicting the likelihood of blood transfusion af-
24. Larrocque BJ, Gilbert K, Brien WF. Prospective valida-
tion of a point score system for predicting blood trans-
25. Goodnough LT, Monk TG, Andreoli GL. Erythropoi-
26. Stowell CP, Chandler H, Jove M, Guillfoyle M, Wa-
choitz M. An open-label, randomized study to com-
pare the safety and efficacy of peripherer epoepoieti-
27. Goodnough LT. Guidelines for the treatment of pre-
epoietin beta (recombinant human erythropoietin) in pa-
apy on platelets and hemoagglutination in patients undergo-
ing cardiac surgery. J Lab Clin Med 1997; 129:376-
83.
gen availability with oxygen status algorithm in pa-
tients undergoing open heart surgery treated with epo-
32. Goodnough LT, Desposit GJ, Parvin, CA. Erythropoi-
33. Gaudiani VA, Monson HDW. Preoperative erythropoi-
34. Monk TG, Goodnough LT, Brecher ME, Colberg JW, Andreole GL, Catalona WJ. A prospective, ran-
domized trial of three blood conservation strategies for radical prostatectomy. Anesthesiology 1999; 91:24-
33.
36. M Canhon F, Vargas R, Ryan M, et. al. Pharmacoki-
etics and efficacy of recombinant human erythro-
ty and efficacy comparison study of two dosing regi-
ners of erythropoietin alpha in patients undergoing major orthopedic surgery. Am J Orthop 1996; 25:544-
52.
38. Goodnough LT. Erythropoietin and iron therapy. In: Spence RK, ed. Problems in general surgery, Lippin-
cott, Williams & Wilkins, Philadelphia, PA, In Press.
39. Welch GH, Meehan KR, Goodnough LT. Prudent strate-
40. Goodnough LT. Blood transfusion and blood conserv-
63.
42. Goodnough LT, Grishaber JE, Monk TG, Catalona WJ. Acute preoperative hemodilution in patients under-
43. Monk TG, Goodnough LT, Brecher ME. Acute nor-
movolemic hemodilution can replace peroperative au-
tologous donation as a method of autologous blood procurement in radical prostatectomy. Anesthesiol


Diagnostic approaches to suspected deep vein thrombosis and pulmonary embolism

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Diagnosis of deep vein thrombosis (DVT) relies mainly on three instruments: venous compression ultrasonography (US), assessment of prior clinical probability (PCP), and measurement of fibrin D-Dimer (DD). In suspected pulmonary embolism (PE), the same tools can be applied, in addition to a ventilation/perfusion lung scan (V/Q scan).

In a few patients, venography (suspected DVT) or pulmonary angiography (suspected PE) may also be required. In order to face the continuous decrease in prevalence of DVT and PE in the suspected populations and to reduce the need for invasive and costly procedures, sequential diagnostic strategies were developed and studied in large outcome studies.

This review will focus on these sequential approaches and, more specifically, on the place of D-Dimer, a degradation product of crosslinked fibrin that is more and more used to rule out DVT and PE in clinically suspected individuals.

Diagnostic approach to suspected DVT

Table 1 compares four recently proposed management strategies.1-4 The first one relies on serial US, i.e., repetition of compression US after one week in all patients with no DVT detected in the initial examination. The second one relies on serial US restricted to patients with a normal initial US and an abnormal DD result (rapid latex test).2 In the third strategy, serial US was restricted to patients with a non-low PCP while in the last algorithm, a single US was performed in patients with a DD result above the critical cut-off (rapid ELISA method).4 PCP allowing identification of patients requiring venography (those with a high PCP, a positive DD and a negative US).

These four strategies were assessed in management trials with long-term (at least three-month) follow-up. In the three trials in which DD,2 PCP3 or both4 were added to the initial US exam, the proportion of patients requiring a repeat US exam at one week was reduced from 76% in the study relying on US alone1 to 9% when the DD latex test was added,2 to 28%3 when PCP was added, or 0% when DD ELISA test and PCP assessment were added4 (Table 1).

Compression venous US appears thus to be the key diagnostic tool in out-patients clinically suspected of having DVT. However, because the prevalence of DVT in the suspected population is quite low (approximately 20%), using a highly sensitive DD (ELISA) test as an initial screening would reduce the number of initial US exams by at least one quarter. Alternatively, PCP or DD may be used to diminish the number of repeat US. However, the principle of repeat US after one week to pick up undiagnosed distal DVT that would have extended proximally, albeit very attractive, has a low yield and may not be very cost-effective. Lastly, the low three-month thromboembolic risks in these four studies (Table 1) were obtained by means of a compression US technique limited to the examination of the common and superficial femoral, and the popliteal veins. This at least questions the affirmation by some investigators that a complete, more time-consuming venous US examination (from the calf to the inferior vena cava) should be performed in all patients clinically suspected of DVT.

Diagnostic approach to suspected PE

PE diagnosis has undergone a true revolution in the past 15 years. One major aspect of this revolution was the recognition that PE and DVT are two clinical manifestations of a single entity, namely venous thromboembolism. This means specifically that diagnosing a DVT in a patient with clinically suspected PE is sufficient to diagnose PE, an attitude that is in keeping with the identical treatment of these two conditions, except for massive PE, a situation which is beyond the scope of this brief review.

Recently, two sequential, mainly non-invasive strategies were applied in large cohorts of patients with suspected PE.4,5 The strategies are based on PCP, assessed empirically4 or by means of a clinical model;5 on a rapid DD ELISA test;4 on venous compression US;4,5 on a V/Q lung scan;4,5 and in some cases, on pulmonary angiography. These strategies are depicted in Figures 1 and 2.5 The three-month thromboembolic risks that were associated with these algorithms were 0.9% (95% CI: 0.2%-2.7%)4 or 0.5% (95% CI: 0.1%-1.3%)5 (5). Pulmonary angiography had to be performed in 11% or 4% of patients. These strategies allow the majority of patients to be managed with widely available, non-invasive diagnostic tools.

These strategies did not include spiral CT scan. Indeed, the rapid acquisition of high contrast images by spiral CT scanning allows good visualisation of the pulmonary arteries down to at least the segmental level, which led to a premature, widespread use of this technique which eventually became the reference diagnostic tool in many institutions. As shown in Table 2, there are wide variations in the reported perfor-
Table 1. Four diagnostic strategies in patients clinically suspected of having DVT.

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<th>Tool/Strategy</th>
<th>Cogo et al.¹</th>
<th>Bernardi et al.²</th>
<th>Wells et al.³</th>
<th>Perrier et al.⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RUS</td>
<td>RUS+DD</td>
<td>RUS+PCP</td>
<td>US+DD+PCP</td>
</tr>
<tr>
<td>N of patients</td>
<td>1,702</td>
<td>946</td>
<td>593</td>
<td>474</td>
</tr>
<tr>
<td>DVT prevalence</td>
<td>24%</td>
<td>28%</td>
<td>16%</td>
<td>24%</td>
</tr>
<tr>
<td>PCP</td>
<td>-</td>
<td>-</td>
<td>score</td>
<td>empirical</td>
</tr>
<tr>
<td>D-dimer</td>
<td>-</td>
<td>Instant-IA</td>
<td>-</td>
<td>Vidas DD</td>
</tr>
<tr>
<td>US</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>73%</td>
</tr>
<tr>
<td>RUS (n, %)</td>
<td>1,302 (76%)</td>
<td>88 (9%)</td>
<td>166 (28%)</td>
<td>0</td>
</tr>
<tr>
<td>Yield of RUS</td>
<td>0.9%</td>
<td>5.7%</td>
<td>1.8%</td>
<td>-</td>
</tr>
<tr>
<td>Venography (n, %)</td>
<td>0</td>
<td>0</td>
<td>33 (6%)</td>
<td>2 (0.4%)</td>
</tr>
<tr>
<td>3-month VTE risk [% (95% CI)]</td>
<td>0.7% (0.3-1.2)</td>
<td>0.4% (0.0-0.9)</td>
<td>0.6% (0.1-1.8)</td>
<td>2.6% (0.2-4.9)</td>
</tr>
</tbody>
</table>

Table 2. Performance of rapid D-dimer assays in patients clinically suspected of having DVT or PE.

<table>
<thead>
<tr>
<th>Method</th>
<th>n (suspected patients)</th>
<th>n (PE/DVT)</th>
<th>Sensitivity % (95%CI)</th>
<th>Specificity % (95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vidas DD*</td>
<td>1316</td>
<td>173/132</td>
<td>98.7 (96.7-99.6)</td>
<td>39.6 (36.5-42.6)</td>
</tr>
<tr>
<td>LIA-test*</td>
<td>971</td>
<td>310/0</td>
<td>99.4 (97.7-99.9)</td>
<td>39.6 (35.9-43.4)</td>
</tr>
<tr>
<td>SimpliRED**</td>
<td>2393</td>
<td>255/234</td>
<td>90.2 (87.2-92.7)</td>
<td>68.5 (66.5-70.6)</td>
</tr>
</tbody>
</table>

Figure 1. Diagnostic algorithm for suspected pulmonary embolism according to Perrier et al. (modified from ref. #4).

The case for DD measurement

When assayed in plasma by a quantitative ELISA or ELISA-derived methods DD levels have been shown to be highly sensitive (about 99%) in acute PE or DVT at a cut-off value of 500 µg/L even if the analysis is restricted to rapid assays, while the rapid whole blood latex assay was a decidedly lower sensitivity (Table 2).

The specificity of fibrin for venous thromboembolism is poor. Indeed, fibrin is produced...
in a wide variety of conditions, including cancer, inflammation, infection or necrosis. Hence, a DD level above 500 µg/L has a poor positive predictive value for PE, and cannot reliably rule in the disease. Moreover, specificity of DD is even lower in the very elderly (less than 10% in suspected PE patients older than 80 years). However, the situation might be different if a higher cut-off (4000 or 10,000 µg/L) were chosen, the likelihood ratio for a patient clinically suspected of PE with a DD level of this order of magnitude being at least 5.

Obviously, the place and utility of DD in a diagnostic strategy will depend upon its sensitivity on the one hand, and upon the prevalence of the disease in the targeted population on the other hand. A highly sensitive assay can be used as an initial screening test allowing venous thromboembolism to be ruled out in about one third of suspected patients but a less sensitive assay can only be used in combination with another test, venous compression US or clinical probability assessment for example. Conclusion and perspectives

Diagnosing DVT and PE has become definitely easier and more reliable over the past fifteen years, especially thanks to the development of venous compression US of lower limbs and DD measurement, which has reduced the requirement for venography and pulmonary angiography to a minority of patients (6% or less, and 10% or less, respectively). The new strategies have proved to be safe and are likely to be cost-effective. They should now be implemented in daily practice. In the near future, spiral CT might be included in the diagnostic approach of PE but its exact place remains to be determined.

References


Figure 2. Diagnostic algorithm for suspected pulmonary embolism according to Wells et al. (modified from ref. #5). PE: pulmonary embolism; US: lower limb venous ultrasonography; DVT: deep vein thrombosis; PCP: prior or clinical probability; near-N: near-normal; Rx: treatment; no Rx: no treatment.
Initial treatment of patients with venous thromboembolism

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* Laboratory for Experimental Internal Medicine; °Department of Clinical Epidemiology & Biostatistics; #Department of Vascular Medicine, Academic Medical Center, Amsterdam, The Netherlands

In recent years suggestive evidence has been collected for the concept that deep venous thrombosis (DVT) and pulmonary embolism (PE) are expressions of a single disease, venous thromboembolism (VTE). The following observations support this assumption. Firstly, it has become clear that about 75% of all patients presenting with either expression of VTE, also have evidence of the disease being present at the other locations. However, until now it remains unclear why one patient presents with pulmonary symptoms, while the other primarily presents with symptoms of the legs. It is even more puzzling when one looks at recurrence, since 80% of the recurrent episodes occur in the same location. Secondly, from epidemiological and laboratory investigations it was concluded that DVT and PE have an almost completely overlapping pathophysiology. Both expressions of thrombosis share the same acquired and transient aetiological risk factors: e.g. immobilisation, surgery, protein C and S deficiencies and the G20210A prothrombin mutation. Recently, it was found that the factor V Leiden mutation has a different prevalence in PE and DVT patients.1 This observation, however, does not refute the concept of DVT and PE being two expressions of the same disease, since this more likely reflects the tendency of embolisation of the clot due to the composition of the clot. Thirdly, the modalities used in the treatment of both DVT and PE are roughly the same and mainly dependent on the extent of the symptoms that the patient presents with.

Both diseases have a clinical spectrum of severity of symptoms. In the case of DVT the patients may present with symptoms differing from small localised calf thrombi, with or without minor complaints, without a tendency to progress via thrombus in the popliteal vein, to extensive DVT with compromised arterial circulation, i.e. phlegmasia cerulea dolens. Patients suffering from PE may also present with a similar spectrum, ranging from patients with or without minimal complaints and self-limiting disease, to patients with haemodynamic shock due to extensive pulmonary obstruction. Analogous to these spectra, the treatment strategies for patients with either DVT or PE may vary. Patients with small, localised thrombi in the calf veins may not need anticoagulant treatment when serial compression ultrasound investigations of the more proximal veins remain normal. The other end of the spectrum consists of patients with extensive DVT, who may benefit from thrombolytic therapy or even a surgical approach, and placement of a caval filter to prevent PE. Patients with small emboli without a tendency to recur might be watched untreated. Patients with extensive PE may suffer from shock and possibly benefit from thrombolytic therapy and even surgery, analogous to patients with DVT. However, the great majority of patients presenting with VTE do not belong to these extremes of the spectrum. Numerous clinical studies have pointed out that patients in the large middle group can safely be treated with initial unfractionated heparin (or in more recent years low molecular weight heparin), followed by a course of at least three months of dose adjusted vitamin K antagonist treatment.

In this review we will assess the trial evidence for the optimal initial treatment of venous thromboembolism, based on the spectrum presented.

Approach to patients with suspected venous thromboembolism

Numerous clinical trials have revealed that when serial compression ultrasound investigations of the more proximal leg veins remain normal in patients presenting with suspected DVT, it is safe to withhold anticoagulant therapy.2,3 Previous venographic studies have shown that some of these patients have localised calf vein thrombosis. As for DVT, in patients with suspected PE it is a safe strategy to withhold anticoagulant treatment when a non-diagnostic lung-perfusion scan is followed by normal serial compression ultrasonography findings.4,5 Several studies have shown that these patients may have a small pulmonary embolism as revealed by angiographic studies. Implicitly these results suggest that it is safe to withhold anticoagulant treatment in patients with DVT or PE who have small, localised thrombi and extension of the disease has been excluded by serial normal testing.

Unfractionated- and low molecular weight heparin in the initial treatment of symptomatic venous thromboembolism

The need for anticoagulant treatment in patients with documented VTE was convincingly established in 1960 in a randomised, placebo-controlled trial conducted by Barritt and Jordan.6 The necessity for and the importance of adequate initial treatment with heparin for approximately 10 days, followed by treatment with vitamin K antagonists for the prevention of recurrent VTE was subsequently estab-
lished in several clinical studies. Others showed that an initial course of 5 days of intravenous unfractionated heparin (UFH) treatment was as effective as a 10-day course. Regardless of the route of administering UFH (intravenous or subcutaneous) the APTT should be prolonged between 1.5 to 2.5 times the control value. Whenever the APTT ratio is below 1.5, the risk of recurrent VTE is increased. However, in a recent meta-analysis of clinical trials that evaluated different UFH regimens in patients with VTE in whom the starting dose was above 30,000 U of UFH per day, the rate of recurrences was not higher in those with initially subtherapeutic APTT levels. Therefore, treatment with intravenous UFH started with a bolus of 5,000 U of UFH, to saturate non-specific heparin binding sites, followed by a continued infusion of 1,250 U of heparin per hour, appears to be the appropriate approach. The dosing of the heparin should be based on regular laboratory measurements of APTT because of the narrow therapeutic window of heparin and the varying response of patients to UFH. It is worthwhile, particularly in under- or overweight patients, to use a weight-adjusted dosage of both the bolus (e.g., 80 U/kg) and the initial infusion rate (e.g., 18 U/kg/hr). Nomograms can be a useful tool for adjusting the infusion rate of UFH to facilitate adequate heparinization.

Low molecular weight heparin (LMWH) has several advantages over UFH. The first is that the half-life of these former compounds is longer, and that their bioavailability is almost 100%, thus allowing them to be given once or twice daily in fixed doses, according to the body weight of the patient, without the need for laboratory monitoring. Furthermore, thrombocytopenia is less of a problem with LMWH.

Recently, Bijsterveld et al. performed a meta-analysis of studies that compared subcutaneous LMWH (once or twice daily) with continuous intravenous unfractionated heparin for the initial treatment of patients with VTE. For the purpose of this review we added recently published studies. Computerised literature searches were performed in EMABSE and MEDLINE to identify additional publications that might describe randomised clinical trials comparing subcutaneous LMWH and continuous intravenous UFH for the initial treatment of VTE. Furthermore, we contacted authors of studies that were only available in abstract form. All these randomised investigations were reviewed. The major outcome measures in the meta-analysis were symptomatic and objectively confirmed recurrent VTE during 3 months of follow-up, major bleeding during the initial treatment period, and death of any cause in the first three months after the initial thrombosis. All studies compared initial treatment lasting 5 to 10 days and all patients thereafter received three months of vitamin K antagonists. We identified three additional studies that satisfied our predefined inclusion criteria, which bring the total number of studies available for analysis to sixteen. In total, approximately 5,000 patients were included in this meta-analysis. We observed a clinically relevant and statistically significant reduction of recurrent VTE in the first three months of follow-up in favor of LMWH (odds ratio (OR) 0.69 (95% CI [0.54, 0.90]) (13 studies were eligible for this outcome) (Figure 1).

The OR for major bleeding (15 studies) was 0.56 in favor of LMWH (95%CI (0.38, 0.84)) (Figure 2), whereas the OR for mortality (11 studies) showed a statistically significant difference in favor of LMWH of 0.67 (95% CI (0.51, 0.87)). In conclusion, the present meta-analysis shows that initial treatment with subcutaneous LMWH (once or twice daily) is more effective in preventing recurrent VTE than UFH. Furthermore, the data indicate that treatment with LMWH is associated with a lower rate of major bleeding complications during the initial treatment phase. The survival advantage in the LMWH group is limited to the subgroup of patients with malignancies. Many hypotheses have been proposed to explain

![Figure 1. Meta-analysis of LMWH versus UFH in the initial treatment of VTE (incidence of recurrent VTE during three months of follow-up).](image-url)
this observation, e.g., influence of these compounds on angiogenesis and tumour progression. The precise mechanism and the real effect remain, however, to be elucidated in further, prospective randomised trials.

All LMWH compounds have similar efficacy and safety compared to UFH. The dosage and the frequency of administration (once or twice daily) of the specific LMWH used, however, should be based on the clinical trial conducted with the specific compound. Confusion may arise when the doses of the various LMWH compounds available are compared. Furthermore, the dose of LMWH should be based on the weight of the patient.

So far three studies have addressed the issue of treating patients with symptomatic PE with low molecular weight heparin. In total, approximately 1,100 patients were included in these studies. These studies showed that LMWH is at least as effective and safe as UFH in the treatment of patients with symptomatic PE. Further trials are needed in this specific patient group.

In general, treatment with vitamin K antagonists should be started at the same time as heparin treatment (UFH or LMWH) is initiated. It is safe to discontinue heparin treatment after at least 5 days when the International Normalized Ratio (INR) is above 2.0, for at least two measurements 24 hours apart.

Outpatient treatment with low molecular weight heparin

The convenience of treating patients with DVT with once or twice daily subcutaneous low molecular weight heparin in hospital raised the question of whether it was feasible to treat patients with LMWH in an outpatient setting. A few issues have to be considered before out of hospital treatment can be widely advocated. Firstly, the efficacy and safety of this treatment needs to be documented in large, prospective clinical studies. Secondly, the cost-effectiveness of home treatment needs to be analysed and finally, adequate programmes for the successful implementation of home treatment should be developed. In studies comparing outpatient treatment of acute DVT with LMWH and in-patient treatment with intravenous UFH, it has been reported that both treatment modalities have a comparable safety and efficacy in the treatment of patients with symptomatic proximal DVT. Similar data have been obtained in several cohort studies. Van den Belt et al. showed that outpatient management of patients with proximal DVT using LMWH reduces resources’ utilisation and total treatment cost, a finding recently confirmed finding. Finally, successful treatment of patients with DVT in an outpatient setting will require intensive patient education, as well as an extensive infrastructure of supportive nursing and physician services. Careful patient follow-up will be crucially important. For patients with PE only very limited data on out of hospital treatment is available. Hence this strategy is still experimental.

Vena caval filters

A recent randomised clinical trial by Decousus and co-workers in approximately 400 patients with proximal DVT compared a permanent caval filter with no filter. All patients received (LMW) heparin and vitamin K antagonists. After 12 days, the patients in the filter group had had fewer (silent) PE than those without a filter (OR 0.22: 95% CI [0.05, 0.90]). Although, the number of PE was lower in the group of patients receiving a filter, after 2 years of follow-up, there was an excess incidence of recurrent DVT in these patients as compared to the no-filter group. No difference was observed in the overall mortality. In conclusion,
based on these limited data, there is not enough evidence to promote routine placement of caval filters in patients with VTE who receive adequate anticoagulant treatment. Placement of a filter should be considered in patients with contraindications to anticoagulation, or patients with recurrent VTE during adequate anticoagulant therapy. However, it should be realised that modern vena caval filters can be taken out after, for instance, 7 days. It is possible that the use of these filters prevents early PE in patients with DVT and does not have long-term disadvantages. Data from randomised clinical trials in this area are needed before this strategy can be recommended.

Thrombolysis

Thrombolytic therapy has been studied for many years and despite this, its role in the treatment of VTE remains questionable. Thrombolytic therapy is now limited to patients with severe symptoms of VTE, i.e. PE with haemodynamic instability or phlegmasia cerulea dolens.

Thrombolysis in deep venous thrombosis

Theoretically, the benefit of thrombolysis in the treatment of DVT lies in the following: rapid resolution of thrombus might give earlier relief from symptoms; the resolution might lead to lesser damage to valves and could result in a decrease of the risk of post-thrombotic syndrome; complete resolution of the thrombus may lead to a lower risk of recurrent VTE. The trial evidence so far indicates that patients treated with thrombolysis have indeed a more rapid resolution of thrombus as compared to patients treated with UFH, at the cost of a four-fold higher risk of major bleeding. Good long-term follow-up information on patients treated with thrombolysis is lacking. However, available studies do not indicate that thrombolysis has an advantage in terms of a lower risk of post-thrombotic syndrome or recurrent DVT. In conclusion, thrombolysis is not a routine treatment for patients with DVT. However, the exception might be for patients with limb-threatening conditions, e.g. phlegmasia cerulea dolens.

Thrombolysis in pulmonary embolism

As for DVT the benefit of thrombolysis may be the rapid restoration of lung perfusion, with secondary better clinical outcome. Several randomised, controlled trials have been conducted that compared the treatment of patients with thrombolysis to treatment with UFH. Although the initial perfusion scans and haemodynamic indicators were indeed significantly better in the thrombolysis group, these studies did not show a significantly better long-term outcome in terms of recurrent PE and mortality. In conclusion, thrombolysis is not the first choice of treatment for patients with PE, except for those with haemodynamic instability.

Patients suffering from PE, complicated by right ventricular failure as revealed by echocardiography, but without signs of haemodynamic impairment, were recently identified as an interesting subgroup. These patients seem to have a higher mortality rate than patients with normal right ventricular function. Some indication exists that these patients might benefit from thrombolytic therapy. This concept, however, needs to be evaluated in well-designed randomised, clinical trials.

Thrombectomy (surgically or percutaneously)

Thrombectomy, either surgically or percutaneously, in patients with PE or DVT is not often used. Thrombectomy failed to show a substantial long-term benefit in single randomised clinical trial in patients with DVT. Furthermore, this treatment modality is complicated by a substantial rate of acute recurrences because damaged venous endothelium is highly thrombogenic. Therefore, thrombectomy is a treatment that should be reserved for those patients who have massive haemodynamic instability due to PE and who are unresponsive to or have absolute contraindications to thrombolytic therapy. The use of catheter thrombectomy or thrombus fragmentation together with local or systemic thrombolysis may be a possible new treatment modality in these patients. This treatment, however, needs to be validated in proper clinical trials.

Conclusions

Patients suffering from either DVT or PE may present with a broad range of symptoms ranging from patients without any symptoms to patients with limb-threatening disease in the case of DVT and haemodynamic instability in those suffering from PE. However, the great majority of patients with VTE are not in the extremes of this spectrum. This clinical spectrum is reflected by modalities used in the treatment of VTE. The majority of patients can safely be treated with a minimum of five days of (LMW) heparin followed by at least a three month course of vitamin K antagonists. Thrombolysis, thrombectomy and the placement of caval filters are treatment modalities for only carefully selected subgroups of patients suffering from VTE, and more, properly designed, randomised trials are needed in order to be able to recommend these treatment strategies to a broader spectrum of patients.

References

 Session 9 – CLINICAL MANAGEMENT OF THROMBOSIS

Prevention of venous thromboembolism in medical and surgical patients

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Cardiovascular Division; Brigham and Women's Hospital, Harvard Medical School, Boston, USA

Successful prevention of venous thromboembolism (VTE) among hospitalised patients serves as a benchmark for delivering high quality care. In North America, a Consensus Conference convened and endorsed by the American College of Chest Physicians (ACCP) has gained tremendous influence over daily clinical practice. This document has also served as a starting point for plaintiffs suing physicians. Plaintiff’s attorneys often claim that the ACCP guidelines constitute de facto standard of care and that physicians are medico-legally liable when the guidelines are not followed and VTE ensues.

European versus North American perspective

In Europe, a separate group undertook a detailed literature review, formulated guidelines, and published their report on prevention of VTE under the auspices of the International Union of Angiology. These guidelines—though well written, logical, and practical—have received very little attention. Most clinicians in North America are unaware of them. A subcommittee of North Americans and Europeans met in Dallas in November 1999 to begin drafting an update of this document. No major philosophical divisions emerged between members based upon country of origin. Nevertheless, a group of prominent European haematologists and vascular specialists perceive that there is a European way of administering prophylaxis and a separate North American approach.

Prophylaxis is like immunisation

I view the concept of prophylaxis as having similarities to principles of immunisation against infectious diseases. Like childhood infectious diseases such as measles, mumps, and rubella, VTE often causes physical discomfort, emotional distress, loss of productivity, and occasionally serious and even fatal complications. VTE has the added problem of often being difficult to detect because it can be asymptomatic or can mimic other disorders. For example, deep venous thrombosis can masquerade as calf muscle strain or cellulitis; pneumonia or congestive heart failure can mimic or obscure the diagnosis of pulmonary embolism. The fundamental difference between VTE prophylaxis and vaccination is that most vaccines are more effective, less expensive, and less complicated to administer than protocols of prophylaxis against VTE.

Encouraging the use of prophylaxis

VTE prophylaxis requires a rather imprecise risk stratification of patients and then a series of decisions concerning the method and duration of the preventive intervention. Accepted prophylactic methods for most indications include anticoagulants, mechanical devices such as graduated compression stockings or intermittent pneumatic compression devices, or a combination of both strategies. The murky business of risk stratification and the wide range of choices available for prophylaxis create a recipe for confusion and an abundance of competing strategies rarely tested in adequately powered head-to-head clinical trials. Unfortunately, prophylaxis is often overlooked entirely, perhaps because of uncertainty about level of risk and optimal prevention strategy.

Another problem is that many of the prophylactic methods introduced a generation ago may not prevent VTE in today’s critically ill patient. Most studies have been undertaken in haemodynamically stable patients with low mortality rates. At Brigham and Women’s Hospital, we intend to test a novel system to encourage institution of prophylaxis in all high-risk patients. Our hospital has a sophisticated electronic tracking system that can determine not only patients’ ages but also whether they have undergone a recent major operation and whether they have a history of cancer and prior VTE. Our orders are always entered electronically, so the computer system can also determine whether patients at high risk for VTE are receiving preventive measures. We plan to identify high-risk patients and randomise them to having their physician receive an electronic warning on electronic mail versus not receiving a warning. Our primary objective will be to determine whether patients randomised to the group that generates warnings have a lower VTE rate than the usual care group.

Medical patients

Historically, medical physicians have chided their surgical colleagues for failure to insist upon adequate VTE prophylaxis for perioperative patients. This stance may never have been justified and certainly is inappropriate in the new millennium. Surgeons have designed and facilitated many more comprehensive and definitive clinical trials among their patients than we internists have undertaken among our patients. Consequently, the rates of major perioperative pulmonary embolism and deep venous thrombosis appear to be plummeting. In fact, at Brigham and Women’s Hospital, General Surgery and Or-
thopaedic Surgery patients account for less than one quarter of the overall group that developed in-hospital VTE. Thus, medical Service patients and especially medical Intensive Care Unit patients remain the last frontier for developing and implementing effective prophylaxis.

Pharmacological prophylaxis

An Israeli study was the first to support the concept that mortality reduction could be achieved in hospitalised general medical patients by using low dose heparin prophylaxis. The study enrolled 1,358 consecutive medical patients more than 40 years of age admitted through the Emergency Department of an acute care hospital. Those with even number hospital records were assigned to low dose heparin, 5,000 units twice daily. Those with odd number records served as controls. Among patients allocated to heparin, there was a 31% reduction in mortality, from 10.9% in the control group to 7.8% in the heparin group. The reduction in mortality in the heparin treated group was evident from the first day, and the difference increased significantly and consistently with time until the end of the study period.

The Royal Infirmary in Glasgow studied 100 medical patients hospitalised with heart failure or chest infection. They were randomised to receive either heparin 5,000 units every 8 hours or to receive no specific prophylaxis measures. The diagnosis of DVT was established by iodine-125 fibrinogen leg scanning, which was undertaken in all study patients within 24 hours of hospitalisation and repeated every other day for 14 days or until hospital discharge. Among controls, 26% developed DVT, whereas the rate was only 4% among those receiving low dose heparin.

Among octogenarian medical inpatients, a placebo controlled randomised double blind study utilized in 1986 a then novel once daily low molecular weight heparin (Pharmuka 10169, subsequently renamed enoxaparin). The dose was 60 mg injected subcutaneously once daily. The potential development of deep venous thrombosis (DVT) was assessed by iodine-125 fibrinogen leg scanning in all patients. The trial lasted 10 days, and 270 patients were enrolled. The majority suffered from heart failure, respiratory diseases, stroke, or cancer. Of 263 evaluable patients, 9% in the placebo group developed DVT, compared with 3% of those receiving enoxaparin prophylaxis. Except for infection site haematomas, bleeding complications were not appreciably increased in the enoxaparin group.

In a megatrial of 11,693 medical patients with infectious diseases, random assignment was made to heparin 5,000 Units every 12 hours or to no prophylaxis. Although patients were treated for a maximum of 3 weeks, follow-up was carried out for a maximum of 2 months. Heparin prophylaxis delayed the occurrence of fatal PE from a median of 12 to a median of 28 days. There were far more non-fatal thromboembolic complications in the control group (116 vs. 70, p = 0.0012). However, for the prespecified primary endpoint of autopsy verified PE, there was virtually no difference between the two groups: 15 heparin treated and 16 control group patients had autopsy proven fatal PEs. This large trial yielded disappointing results and may have been flawed by: 1) lack of power to detect a difference in the primary endpoint, 2) restriction of heparin prophylaxis to 3 weeks (despite follow-up for 2 months), and 3) an inadequate dose of twice daily heparin. (The International Multicentre trial gave prophylaxis with low dose heparin every 8 hours, not every 12 hours.)

The MEDENOX trial of enoxaparin versus placebo prophylaxis in medical patients randomised to the patients one of three treatment groups in a double blind controlled trial: 1) enoxaparin 20 mg once daily, 2) enoxaparin 40 mg once daily, or 3) placebo. The principal endpoint was the incidence of DVT as assessed by contrast venography between days 6 and 14. Those receiving enoxaparin 40 mg once daily had a two-thirds reduction in DVT compared with those receiving placebo or 20 mg daily of enoxaparin (Table 1).

The Veterans Affairs Cooperative Studies Program has initiated a randomised trial of low dose heparin prophylaxis and potential reduction of mortality among hospitalised general medical patients. Results will be available in about four years.

Mechanical prophylaxis

Intermittent pneumatic compression devices constitute an alternative non-pharmacological approach to prevent VTE. Though effective, special care must be taken to ensure that these devices are worn as prescribed. Frequent removal and non-use can be problematic, especially among patients well enough to sit in a chair or walk. In addition to the mechanical effect of increasing venous blood flow in the legs, these devices increase endogenous fibrinolysis by stimulating the vascular endothelial wall.

Medical intensive care unit

Medical Intensive Care Unit patients pose special challenges which may impede plans for prophylaxis. First, these patients are often bleeding overtly or are admitted with thrombocytopenia. Accordingly, heparin or warfarin are often contraindicated. Second, the presence of leg ulcers, wounds, or peripheral ar-

Table 1. Detailed efficacy results from the MEDENOX Trial.

<table>
<thead>
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<th>Placebo</th>
<th>Enoxaparin 20 mg</th>
<th>Enoxaparin 40 mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients evaluated</td>
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<td>287</td>
<td>291</td>
</tr>
<tr>
<td>VTE</td>
<td>43 (14.9%)</td>
<td>43 (15.0%)</td>
<td>16 (5.5%)</td>
</tr>
<tr>
<td>- All DVT</td>
<td>41 (14.3%)</td>
<td>43 (15.0%)</td>
<td>16 (5.5%)</td>
</tr>
<tr>
<td>- Proximal DVT</td>
<td>14 (4.9%)</td>
<td>13 (4.5%)</td>
<td>5 (1.7%)</td>
</tr>
<tr>
<td>- PE</td>
<td>2 (0.7%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Secondary outcome (day 1-110)</td>
<td></td>
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</tr>
<tr>
<td>Number of patients evaluated</td>
<td>263</td>
<td>263</td>
<td>272</td>
</tr>
<tr>
<td>VTE</td>
<td>45 (17.1%)</td>
<td>46 (17.5%)</td>
<td>19 (7.0%)</td>
</tr>
<tr>
<td>- All DVT</td>
<td>42 (16.0%)</td>
<td>45 (17.1%)</td>
<td>17 (6.2%)</td>
</tr>
<tr>
<td>- Proximal DVT</td>
<td>17 (6.5%)</td>
<td>14 (5.3%)</td>
<td>6 (2.2%)</td>
</tr>
<tr>
<td>- PE</td>
<td>2 (0.8%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
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Table 2. Prevention of VTE among surgical patients.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Prophylaxis Strategy</th>
</tr>
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<tbody>
<tr>
<td>General Surgery</td>
<td>UFH 5,000 U bid/tid or Enoxaparin 40 mg sc q 24h or Dalteparin 2,500 or 5,000 U sc q24h *Nadroparin 3,100 U sc q24h *Tinzaparin 3,500 U sc q24h ± GCS</td>
</tr>
<tr>
<td>Total Hip Replacement</td>
<td>Warfarin (target INR 2.5) or IPC or Enoxaparin 30 mg sc bid or Danaparoid 750 U sc bid</td>
</tr>
<tr>
<td>Total Knee Replacement</td>
<td>Enoxaparin 30 mg sc bid or Ardeparin 50 U/ kg sc bid</td>
</tr>
<tr>
<td>Neurosurgery</td>
<td>GCS + IPC ± UFH 5,000 U bid or Enoxaparin 40 mg once daily (25)</td>
</tr>
<tr>
<td>Trauma (non-brain)</td>
<td>Enoxaparin 30 mg sc bid</td>
</tr>
<tr>
<td>Thoracic Surgery</td>
<td>GCS + IPC + UFH 5,000 U tid</td>
</tr>
<tr>
<td>Radical Prostatectomy</td>
<td>Warfarin (target INR &gt;1.5) plus IPC (26)</td>
</tr>
<tr>
<td>Uncomplicated CABG</td>
<td>GCS±UFH 5,000 U bid/tid or IPC</td>
</tr>
</tbody>
</table>

References

4. Goldhaber SZ, Dunn K, MacDougall RC. New onset of venous thromboembolism among hospitalized patients at Brigham and Women’s Hospital is caused more often by failure rather than by withholding prophylaxis. Personal communication.


β thalassaemia

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β thalassaemia belongs to the family of inherited haemoglobin disorders characterised by a quantitative reduction of functional β globin chains. Although it is defined as a quantitative deficiency of β globin production, some forms result from structural haemoglobin variants that are ineffectively synthesised or are so unstable that they result in a deficiency of the β chains and a thalassaemia phenotype.

The β globin gene cluster

The human β globin complex spans a region of 70 kb on the short arm of chromosome 11 (11p15.15) and contains five functional genes (5'→Gγ-Aγ-Ψβ-δ-β-3') which are arranged in the order of their developmental expression.

The general structure of the β globin gene is typical of other globin loci (Figure 1). The genomic sequence which codes for 146 amino acids spans 1600 bp, and is divided into three exons (coding regions) by two non-coding intervening sequences (introns or IVS). Exon 2 encodes the residues involved in haem binding and αβ dimer formation, while many of the amino acids involved in globin subunit interactions required for the Bohr effect, and 2,3-DPG binding, are found in exon 3. Conserved sequences important for gene function are found in the 5' promoter region, at the exon-intron junctions, and in the 3' untranslated region (3'UTR) at the end of the mRNA sequences. The β globin gene promoter includes 3 positive cis-acting elements: TATA box (positions -28 to -31), a CCAAT box (positions -72 to -76) and duplicated CACCC motifs (proximal at positions -86 to -90, and distal at position 101 to -105). While the CCAAT and TATA elements are found in many eukaryotic promoters, the CACCC sequence is found predominantly in erythroid cell-specific promoters. Binding of erythroid Krüppel like factor (EKLF) to the CACCC motif appears crucial for normal adult β globin expression. The importance of these various 5'-flanking sequences for normal gene expression is underscored by β thalassaemia arising from point mutations in these sequences specifically in and around the TATA box and the CACCC motifs in the -80 to -100 region. An enhancer is also found in intron 2 and 3 of the β globin gene, 600 to 900 bp downstream of the poly (A) site.

Upstream of the entire β complex is the β locus control region (β LCR) which consists of five DNase I hypersensitive sites (designated HS 1-5) distributed between 6 to 20 kb 5' of the ε globin gene. The LCR plays a critical role in globin gene expression; it maintains an 'open' globin locus domain and acts as a powerful enhancer of globin gene transcription, in the absence of which the level of gene expression is low. Four of the sites (HS 1-4) are erythroid specific, encompassing binding sequences for erythroid-restricted transcription factors (GATA-1 and NF-E2), while HS5 is ubiquitous. While the α-like genes undergo a single developmental 'switch' (embryonic → fetal/adult), the β-like genes undergo two 'switches' (embryonic → fetal → adult). Transcription of the ε gene in the embryonic yolk gene switches after the 6th week of gestation to the transcription of the two γ genes in the fetal liver, and then around the prenatal period, to that of the δ (minor adult) and β (major adult) genes. At 6 months after birth, Hb F comprises less that 5% of the total haemoglobin and continues to fall reaching the adult level of <1% at 2 years of age. It is at this stage that mutations affecting the β gene become clinically apparent.

The tissue and developmental specific expression of the individual globin genes is governed by direct physical interactions between the globin promoters and the LCR, the interaction mediated through binding of tissue restricted and ubiquitous transcription factors. This precise developmental expression relies on two mechanisms - gene silencing and gene competition. While the ε and γ globin genes are autonomously silenced at the appropriate developmental stage, expression of the adult β globin gene depends on the lack of competition from the γ gene for the LCR sequences.

Molecular basis of β-thalassaemia

Almost 200 β thalassaemia alleles have now been characterised. Mutations causing β thalassaemia result in either a complete absence, ββ thalassaemia, or a deficiency of β chains, β+ thalassaemia. Unlike the α thalassaemias, the vast majority of β thalassaemias are caused by point mutations within the gene or its immediate flanking sequences. A few β thalassaemia mutations which segregate independently of the β globin cluster have been described in several families; in such cases trans-acting regulatory factors are presumably involved.
Gene deletions and insertions

Upstream deletions

Three deletions, described in families of Dutch, English and Hispanic origin, are of particular interest because they remove substantial regions of the 5’ end of the cluster but leave the β9252 gene itself intact and yet result in a β9252 thalassaemia phenotype. These deletions silence the β globin gene because they remove all or a substantial proportion of the regulatory elements in the β9252 LCR. While deletion of the three 5’ HS (HS2-4) inactivates the β9252 gene, a family study showed that deletion of the 3’ most LCR element (HS1) does not affect the activity of the β9252 gene.8

Deletions involving only the structural β globin gene

Fourteen detections affecting only the β globin gene have been described. They range in size from 390 bp to >60 kb. Of these, only the 618 bp deletion at the 3’ end of the β gene is common, but even that is restricted to the Sind and Punjab populations of India and Pakistan where it accounts for ~20% of the β thalassaemia alleles. The other deletions, although extremely rare, are of particular functional and phenotypic interest because they are associated with an unusually high level of Hb A2 in heterozygotes. These deletions differ widely in size, but remove in common α region (from positions -125 to +78 relative to the mRNA cap site) in the β promoter which includes the CACC, CCAAT and TATA elements. The mechanism underlying the markedly elevated levels of Hb A2 and the variable increases in Hb F in heterozygotes for these deletions appears to be related to the removal of the 5’ promoter region of the β gene. This removes competition for the upstream LCR leading to its increased interaction with the γ- and δ-genes in cis, enhancing their expression. Although the increases in Hb F are variable, and moderate in heterozygotes, they are adequate to compensate for the complete absence of β globin in homozygotes for these deletions. This mechanism may also explain the unusually high Hb A2 levels which accompany point mutations affecting the promoter regions.9

An insertion involving the globin genes

Transposable elements may occasionally disrupt human genes and result in their inactivation. The insertion of such an element, a retrotransposon of the family called L1, has been reported with the phenotype of β thalassaemia. Despite the insertion of 6-7 kb DNA into its IVS2, the affected gene expresses full length β9252 globin transcripts at a level corresponding to about 15% of normal β9252 globin mRNA.10

Non-deletion forms of β thalassaemia

These defects account for the vast majority of the β thalassaemia alleles (Figure 1). They result from single base substitutions, small insertions or deletions within the gene or its immediate flanking sequences. They may affect any level of gene expression and are classified according to the mechanism by which they affect gene function: transcription, RNA processing or RNA translation.
Mutations affecting transcription
A group of mutations involves single base substitutions in the conserved DNA sequences that form the β globin promoter. Transient expression systems show that they result in an output of β globin mRNA ranging from 10 to 25% of normal, a level compatible with the relatively mild phenotype of these β* thalassaemia. The C-T mutation at position -101 to the β globin gene appears to cause an extremely mild deficit of β globin such that it is ‘silent’ in heterozygotes who have normal Hb A2 levels and normal red cell indices.

Several mutations in the 5'UTR have been characterised since the original CAP +1 A-C allele. They include single base substitutions and minor deletions distributed along this stretch of 50 nucleotides. As in the case of the -101 C-T mutation, heterozygotes for this class of mutations are ‘silent’; the extremely mild phenotype is exemplified in a homozygote for the CAP+1 A-C mutation which has the haematological phenotype of a thalassaemia carrier.

Mutations affecting RNA processing
Intervening sequences must be precisely removed from the precursor mRNA and the coding regions spliced to produce functional mRNA. Mutations that affect either of the invariant dinucleotides (GT at 5'→AG at 3') in the splice junction completely abolish normal splicing and produce the phenotype of β* thalassaemia. Mutations within the consensus sequences at the splice junctions reduce the efficiency of normal splicing to varying degrees and produce a β* thalassaemia phenotype that ranges from mild to severe. For example, mutations at position 5 IVSI, G→C, T or A, considerably reduce splicing at the mutated donor site. On the other hand, the substitution of C for T in the adjacent nucleotide, IVSI position 6, only mildly affects normal RNA splicing even though it activates the same three cryptic donor sites as the IVSI-5 mutations.

Several β thalassaemia splicing mutations involve base substitutions within introns rather than consensus splice sites. The first to be characterised was a G→A substitution at position 110 of IVS1. This region contains a sequence similar to a 3' acceptor site although it lacks the invariant AG dinucleotide. The change at position 110 creates an AG with the result that 90% of the RNA is aberrantly spliced to the new acceptor site. Since only a small amount of normal β mRNA is produced, the phenotype is a severe β* thalassaemia.

Three β thalassaemia alleles have substitutions within IVS2 (positions 654, 705 and 745) that create new donor sites and activate a common cryptic acceptor site at position 579 of IVS2 such that the normal 5' donor site at exon 2/IVS2 is spliced to the activated site at position 579 while the newly created donor site is spliced to the normal 3' acceptor site at IVS2/exon 3 resulting in the retention of part of IVS2 in the misspliced β mRNA. Variable amounts of splicing from the normal donor to the normal acceptor also occur, resulting in phenotypes that range from β* to β* thalassaemia.

Cryptic splice sites are also found within exons, one of which contains the sequence GT GGT GAG G spanning codons 24 to 27 in exon 1. Three mutations within this region activate this cryptic site which acts as an alternative donor site in RNA processing. The codon 24 GGT → GGA substitution is translationally silent while codon 26 GAG → AAG and codon 27 GCC → TCC result in the variants β* and β* respectively. These mutations lead to only a moderate reduction of the normally spliced β globin mRNA which encodes for the particular β globin chain variants; hence, the associated mild β thalassaemia.

Four nucleotide substitutions and two minor deletions affecting the polyadenylation signal (AATAAA) have been described. Other mutations in the 3' UTR include a C→G substitution at nucleotide 6 and a 13 bp deletion at nucleotides 90 downstream of the termination codon.

Mutations affecting RNA translation
Mutations which are expressed at the level of mRNA translation involve either the initiation or extension phases of globin synthesis. Seven single base substitutions affecting the initiation codon (ATG) have been described and all produce β* thalassaemia.

Approximately half of the β thalassaemia alleles are characterised by premature termination of β chain extension causing β* thalassaemia. They result from the introduction of premature termination codons due to frameshifts or nonsense mutations and nearly all terminate within exon 1 and 2. Mutations that result in premature termination early in the sequence (in exons 1 and 2) are associated with minimal steady-state levels of β mRNA in erythroid cells, due to an accelerated decay of the abnormal mRNA referred to as nonsense-mediated mRNA decay (NMD).11 In heterozygotes for such cases, no β chain is produced from the mutant allele and only half the normal β globin is present, resulting in a typical asymptomatic phenotype. By contrast, mutations that produce in-phase termination later in the β sequence, in exon 3, are associated with substantially reduced levels of abnormal β mRNA, comparable to the normal allele which, presumably, translates into variant β chains. These mutants are usually dominantly inherited (see later).

Correlation between phenotypes and genotypes
The clinical manifestations of β thalassaemia are remarkably heterogeneous and are related at the primary level to the degree of globin chain imbalance. Traditionally, the phenotypes of β thalassaemia can be classified into three groups: thalassaemia major (TM), thalassaemia intermedia (TI) and thalassaemia trait (TT) but these are generalisations; often there is much overlap between the groups.

Thalassaemia major
Thalassaemia major describes the severe transfusion-dependent anaemia that results from the inheritance of two β thalassaemia genes, compound heterozygous or homozygous states. Apart from the nature of the β thalassaemia mutation itself, major modulating factors include co-inheritance of α thal-
Thalassaemia and co-inheritance of an inherent ability to increase Hb F response; the genetic determinant may be linked or unlinked to the β globin cluster.

β thalassaemia trait

β thalassaemia trait is usually associated with the inheritance of a single β thalassaemia allele, whether β or β+, and characterised by a mild anaemia with hypochromic microcytic red blood cells, elevated levels of Hb A2 and variable increases of Hb F (up to 2.0%). Globin chain biosynthesis shows α chain in excess of about two-fold. Factors which may modify thalassaemia include the nature of the β thalassaemia allele itself, co-inheritance of α thalassaemia or extra α globin genes (triplicated or quadruplicated α genes - aavo/a, aavo/a).

Rarely, the phenotype of β thalassaemia trait results from homozygosity or compound heterozygosity for very mild or ‘silent’ β thalassaemia alleles. The ‘silent’ β thalassaemias cause only a minimal deficit of β globin production and do not produce a detectable haematological phenotype when present in a single copy, the only abnormality is a very mild imbalance of globin synthesis. ‘Silent’ β thalassaemia alleles include the C→T mutation at position -101 to the β gene, some of the promoter mutations, and those affecting the 5′ and 3′ UTR.

Although the increases in Hb A2 levels in β thalassaemia trait are remarkably uniform (3.5-5.5% of total haemoglobin) unusually high levels as well as normal levels of Hb A2 have been observed. Unusually high levels of Hb A2 of >6.5% seems to be the hallmark of a subgroup caused by deletions or point mutations that affect the TATA box and other regulatory elements in the β promoter.

It has been customary to classify β thalassaemia trait with normal Hb A2 levels into type 1 or silent carrier in which there are minimal or no haematological abnormalities, and type 2, in which the haematological profile is typical of heterozygous β thalassaemia except for the normal level of Hb A2. This phenotypic classification is now of limited use following the characterisation of the truly ‘silent’ β thalassaemia alleles and the realisation that most forms of normal A2 β thalassaemia result from the co-inheritance of δ thalassaemia, either in cis or trans to the β thalassaemia gene. Examples of the latter include: δα CD59 (-A) in cis to βKrossos; δ+27 G→T in cis and in trans to βIVS2: 745 C→G.

Another fairly common cause of normal Hb A2 β thalassaemia in the Greek population is the Corfu form of δβ thalassaemia which removes 7.2 kb DNA from 3′ of δβ gene to IVS2 of the δ gene. The β gene in cis is downregulated by a G→A mutation in position 5 of IVS1. The two lesions in the Corfu δβ thalassaemia have also been described as separate mutations in two different population groups, the 7.2 kb deletion in an Italian and the βIVS1:5 G→A mutation in Algerians.

Normal Hb A2 β thalassaemia should also include deletions of the entire β globin complex (the εγδβ thalassaemias) as well as the upstream deletions which involve the LCR.

Intermediate forms of β thalassaemia

Thalassaemia intermedia includes patients with a broad spectrum of phenotypes which range from a relatively severe anaemia requiring intermittent transfusions to an asymptomatic condition diagnosed by a chance haematological condition. The underlying genotypes are equally heterogeneous resulting from the interactions of other genetic variables with the inheritance of a single or two β thalassaemia alleles (Table 1).

It is important to note that the interacting genetic factors are not mutually exclusive. Co-inheritance of the heterozygous state of α+ thalassaemia has very little effect on homozygous β- thalassaemia while individuals with two α gene deletions and homozygous β+ thalassaemia may have a mild form of β thalassaemia intermedia requiring intermittent transfusion.

The role of increased Hb F response as an ameliorating factor becomes evident in the group of TI patients who are mildly affected despite being homozygous or compound heterozygous for severe β- or β+ thalassaemia and without α-thalassaemia. Although presence of the in cis Xmn I-γ site is a modulating factor, clearly there are some patients who have enhanced Hb F response despite being Xmn I-γ/-. Unfortunately, detection of an inherent capacity for increased Hb F production is presently difficult and usually deduced from family studies.

Inheritance of single copies of β thalassaemia gene can also cause thalassaemia intermedia. In the majority of cases this is related to an increased α globin production from the co-inheritance of triplicated or quadruplicated α genes. The resulting phenotype is dependent on the number of the extra α globin genes.

### Table 1. Molecular basis of β thalassaemia intermedia.

<table>
<thead>
<tr>
<th>1. Homozygous or compound heterozygous state for β thalassaemia</th>
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<tbody>
<tr>
<td>i) inheritance of mild β+ thalassaemia alleles</td>
</tr>
<tr>
<td>e.g. βIVS1: 6 T-C, β promoter mutations</td>
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<tr>
<td>-101 C-T, 5′ UTR and 3′ UTR mutations</td>
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<tr>
<th>ii) co-inheritance of α thalassaemia</th>
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<tr>
<td>effect more evident in β+ thalassaemia</td>
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<tr>
<th>iii) β thalassaemia with elevated γ chain production</th>
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</thead>
<tbody>
<tr>
<td>polymorphism at position -158 -γ gene (Xmn I-γ site)</td>
</tr>
<tr>
<td>γ promoter mutations (rare)</td>
</tr>
<tr>
<td>heterocellular HPFH</td>
</tr>
<tr>
<td>X-linked, Gα-linked</td>
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<thead>
<tr>
<th>2. Compound heterozygotes for β thalassaemia and deletion forms of HPFH or δβ thalassaemia</th>
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</thead>
<tbody>
<tr>
<td>3. Compound heterozygotes for β thalassaemia and β chain variants, e.g. Hb E/β thalassaemia</td>
</tr>
<tr>
<td>4. Inheritance of deletion forms of β thalassaemia which remove the 5′ promoter region</td>
</tr>
<tr>
<td>5. Heterozygotes for β thalassaemia</td>
</tr>
<tr>
<td>i) co-inheritance of extra α globin genes</td>
</tr>
<tr>
<td>(αα/α β of extra/extra)</td>
</tr>
<tr>
<td>ii) dominantly inherited forms of β thalassaemia due to hyper-unstable variant β chains</td>
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</table>
and the nature of the β thalassaemia mutation itself, ultimately relating to the critical threshold of α globin excess above which clinical symptoms develop.

Dominantly inherited β thalassaemia

Some forms of β thalassaemia are dominantly inherited in that inheritance of a single β thalassaemia allele, in the presence of a normal complement of α genes, results in a clinically detectable phenotype. Apart from the usual features of heterozygous β thalassaemia, such as increased levels of Hb A2 and increased α/β globin chain biosynthesis ratios, this group of disorders is also characterised by the presence of large intra-erythroblastic inclusions and the term ‘inclusion body β thalassaemia’ was proposed. However, since similar intra-erythroblastic inclusions are also found in all severe forms of β thalassaemia, this term does not seem appropriate.

More than 30 dominantly inherited β thalassaemia alleles have now been described (Figure 1); they include missense mutations, minor deletions leading to the loss of intact codons, frameshifts arising from minor insertions and deletions, resulting in elongated β variants with abnormal carboxy terminal ends and truncated β variants resulting from nonsense mutations. Unlike the more common recessively inherited forms which lead to a quantitative reduction in normal β globin, they result in the synthesis of β chain variants which are so unstable that in many cases, they are not detectable and only implied by the DNA sequence. The predicted synthesis is supported by the presence of substantial amounts of abnormal β mRNA in the peripheral reticulocytes, comparable in amounts to that produced from the normal β allele.

As discussed previously, the in-phase termination mutations associated with dominantly inherited β thalassaemia all terminate in exon 3 or beyond while the vast majority of the in-phase termination mutations that are recessively inherited lead to termination in exon 1 or 2 (Figure 1). These in-phase termination mutations exemplify how shifting the position of a nonsense codon can alter the phenotype of recessive inheritance caused by haplo-insufficiency, to a dominant negative effect due to the synthesis of an abnormal and deleterious protein.

It is quite clear that the cellular pathology underlying this group of β thalassaemias is related to the synthesis of highly unstable β globin chains which fail to form functional tetramers and precipitate intracellularly, with concomitant redundant α chains, accentuating the ineffective erythropoiesis. Immunoelectron microscopy studies have confirmed that the inclusions bodies derived from the red cells of patients with mutations of this class contained both precipitated α- and β-globin chains. In contrast, the intra-erythroblastic inclusions found in homozygous β thalassaemia consisted only of precipitated α globin.

The dominantly inherited β thalassaemia resemble the intermediate forms of β thalassaemia in their ineffective erythropoiesis; but they also have features of congenital haemolytic anaemias in that, there is a variable amount of peripheral haemolysis. Unlike recessive β thalassaemia which is prevalent in malaria-endemic regions, dominant β thalassaemias are rare, occurring in dispersed geographical regions where the gene frequency for β thalassaemia is very low. The vast majority of the dominant β thalassaemia alleles have been described in single families, many as de novo events. It is likely that the low frequency of the dominant β thalassaemia alleles is due to the lack of positive selection that occurs in the recessive forms.

Clinically, since spontaneous mutations are common in dominant β thalassaemia, it is important that the disorder should be suspected in any patient with a thalassaemia intermedia phenotype even if both parents are haematologically normal and the patient is from an ethnic background in which β thalassaemia is rare.

Genetic modifiers of secondary disease

While the severity of ineffective erythropoiesis and disease in β thalassaemia is primarily related to the degree of α/β globin imbalance, a number of factors may modify the clinical manifestations at the secondary level. The genetic modifiers include: the A(TA); TAA variant in the promoter of the UGT-1A gene and jaundice; genetic variants of the vitamin D receptor, oestrogen receptor and COL1A1 gene promoter and bone disease; mutations of the HFE gene and iron loading; inheritance of the apolipoprotein E (APOE) ε4 allele and cardiac failure.

Acknowledgements

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References


Haemoglobin variants, sickle cell pathophysiology and diagnosis

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Haemoglobin gene variations can interfere in the pathophysiology and diagnosis of sickle cell anaemia in a variety of ways.

One particularly important issue is to avoid erroneous diagnoses, especially those leading to inaccurate genetic counselling. At the same time, biological and clinical phenotypes of sickle cell anaemia can be modulated by some of these same haemoglobin variants.

### Diagnosis confounders

**Heterozygotes**

HbS concentration.

It is well known that α thalassaemia lowers the HbS concentration in AS individuals; in contrast β globin overexpression (α gene triplication) increases the concentration. However the percentage of HbS rarely exceeds 48% in supposedly AS individuals. When the percentage of HbS does exceed 48% (without recent prior transfusion), three hypotheses must be investigated.

1. **The allelic “A” β globin gene has diminished expression, i.e. β thalassaemia trait.** Most often this is easily characterized because HbA percentage is much lowered and HbA2 percentage as well as family data are conclusive. It is less evident when silent β thalassaemia alleles, such as the 101 C→T mutation, are involved. Although not well described this type of interaction is prone to increase the HbS percentage to over 50%. To the same group, we can aggregate the various forms of β persistence of HbF synthesis in δ-delta HbS β globin gene. In fact, in this heterogeneous group, if the HbA percentage is lower than the HbS percentage, the latter is lower than the HbA + HbF percentage, resembling simple HbS trait.

2. **The allelic “A” β globin gene bears a destabilising mutation, i.e. unstable Hb (UH) trait.** Many of these UH variants are electrophoretically similar to HbA and are mildly unstable so that the UH (pseudo A) level is slightly reduced. The instability must not only be carried out under rigorous technical conditions, but also taking in account the presence of HbS which precipitates faster than HbA and might be confused with UH variants with mild instability. S/UH compound heterozygosity may also be suspected when the haematological investigations highlight a chronic haemolytic state with Heinz bodies. Other haemoglobin study methods, such as isoelectric focusing (IEF), urea-triton polyacrylamide gel electrophoresis (UT-PAGE) and reversed phase high pressure liquid chromatography (RP-HPLC), may also be helpful. The prototype of this genetic condition is HbS/Hb Saki (14 Pro).

3. **the coexistence of an α globin variant co-migrating with HbS.** Using standard procedures, the αβγδ% is apparently diminished and the HbS percentage is falsely increased by the addition of the αβγδ% compound. However, this situation is normally easy to diagnose because there is an abundant, abnormally migrating (slow and near HbA2) Hb fraction reflecting the addition of the α and β variant charge differences (αβγδ%).

**Heterozygotes**

2) **Clinical phenotype**

Unless subjected to very extreme environmental or physical conditions HbS trait carriers do not suffer from painful vaso-occlusive crises (VOC) in their musculoskeletal system or spleen. However, it is very common to question whether painful manifestations in an AS individual may be related to a sickle genotype. Most often the answer is no. When documented clinical events, such as splenic infarction, are investigated, an important clue is the finding of signs of haemolysis.

Furthermore, when a few sickle cells are discovered in the blood cell smears the investigators should try to more fully characterize the HbS or HbA structure and genetic factors that may favour red cell sickling.

**a) HbS variants.** βS globin genes with a second mutation inducing an electrophoretically silent amino acid change and enhancing Hb polymerisation may be implicated. Hb S Antilles βVal-623 Ile is such a case in which affected individuals have chronic well compensated haemolysis, an enlarged spleen, splenic infarctions and/or sequestration and recurrent painful bone VOC. The simplest way to detect HbS Antilles is to combine standard characterisation with IEF and UT-PAGE. Almost 50 HbS Antilles carriers have been identified in the last 10 years in France. Other analogous genotypes are probably involved in similar clinical settings. Heterozygous or homozygous HbS Oman gives rise to a moderate sickle cell disease. However, this variant is easily characterised by its biological properties.

**b) HbA variants.** The HbS polymer is a complex tridimensional molecular assembly in which many inter HbS and HbA tetramer contacts involve surface amino acids, the mutation of which could reinforce the co-polymer properties of HbA. Again there is a prototypic case, i.e. Hb Quebec-Chori (β87 Ile) found in a pseudo-A5 individual suffering from a rather severe haemolytic sickle cell disease.
c) Others factors. Elevation of the mean corpuscular haemoglobin concentration (MCHC) due to red blood cell (RBC) water loss and low 2.3 DPG increases are circumstances associated with VOC and spleen infarcts in some rare individuals. Red cell membrane defects can lead to RBC water loss and pyruvate kinase deficiency to high 2.3. DPG concentrations.

Heterozygotes

3) S-like variants. Some Hb variants share with HbS one or other of its in vitro properties without, however, having deoxytetramer polymerisation capacity. The most trivial circumstance is HbS diagnosis based only on cellulose acetate electrophoresis without any necessary confirmatory test such as the red cell sickling test (Emmel test), solubility test (Itano), or citrate agar electrophoresis. Unfortunately, this still often happens, although it does not allow differential diagnosis from the many kinds of HbD or G (which are very common).

Even using the double electrophoretic approach, as recommended (cellulose acetate + citrate agar) some Hb variants can be misdiagnosed as HbS: HbG Makassar (6-Ala); Hb Hirose (37 Ser); Hb Summerhill (52 His); Hb Tak (C-term elongated); Hb Hasharon (47 hist); Hb Lukes (95 Arg); Hb Russ (51 Arg); HbQ India (64 His); Hb Oottawa (15 Arg); Hb Casamance (4 Arg); Hb Handworth (18 Arg); Hb M emphasis (23 Gin); Hb Queens-Oct (34 Arg).

The α globin variants listed above can be distinguished by two features: the observation of a split Hbaα (the slowest migrating fraction being the hybrid αsβδ) and a low levels of expression (10 to 25%) which is uncommon for a HbS trait (except in transfused patients or the very rare coexistence of α haemoglobin H disease). However, the α chain variant can be expressed, at more than 30% when associated with α thalassaemia or in their homozygous form. The specificity of the diagnostic approach is better when cellulose acetate electrophoresis is replaced by IEF but Hb G Makassar, Hb Hasharon, Hb Casamance, Hb Oottawa are still difficult to distinguish from HbS.

This is the reason why the Itano test must always be done as a confirmatory test. Even then, Hb G Makassar remains indistinguishable from HbS.

Electrophoreses in denaturing conditions, particularly UT-PAGE are very powerful techniques for fully characterise these Hb variants morefully. DNA studies, using PCR based methods can very specifically establish the presence of the βS mutation, although the result might remain doubtful with the HbS Makassar mutation.

DNA sequencing and protein structural studies have both advantages and disadvantages and, in some Hb variants with post-translational modifications, gives complementary valuable discriminant information.

Homozygotes

1) Neonatal detection of sickle cell disease (SCD) has been, since 1999, included in the National French Programme for Detection of Genetic Deficiencies at birth. A preliminary diagnosis of SCD can be made accurately by either IEF or HPLC at birth. Confirmatory tests are citrate agar electrophoresis and family studies. False positive results (2 to 4%) comprise three categories of compound heterozygosity.

a) S/Hope in which Hb Hope is like acetylated HbD if IEβ and like HbD in HPLC. The βthalassaemia Short Program (BIORAD) HPLC detects even low quantities of Hb Hope. S/Hope, a rather common genetic condition in west Africans, is absolutely benign.

b) S/Hb Bougardirey-Mali has been found in a few instances; Hb Bougardirey-Mali (β119 Val) is like HbD in IEβ but like HbA in HPLC or CAE. The affected individuals have a chronic haemolytic anaemia without VOC.

c) S/HPHF of the βS category. This is a benign clinical state which is easily detected by family study.

2) Other interferences. Compound heterozygotes for HbS and any kind of HbD are not rare. Most of them are easily characterised by IEF and/or CAE, HPLC. The difficulty is to identify the HbD Punjab (β121 Gin) trait which causes severe sickle cell anaemia in combination with HbS. While UT-PAGE gives a fast presumed identification, the definite diagnosis can be made by simple DNA PCR-based studies.

Combinations of S/D Korfe-Bu or S/D Iran are clinically silent and frequently observed in West Africa. In contrast, HbS Antilles/HbS association is extremely severe but has been observed in only one Martinique family. The HbS/Hb Lepore association has been described a dozen times. It is a very mild SCD easily characterised by IEF and family study. CAE gives an "AS" picture with low "A". HPLC is also very suggestive, provided the sample is in a correct state for analysis.

Interferences of HbS homozygosity with α globin variants are common too. The given electrophoretic phenotype is variable. The size of the fraction bearing the α chain mutation depends on the α1 or α2 nature of the α globin gene concerned and on the possible co-existence of an αα thalassaemia trait; fast migrating α chain variant can mimick HbA or HbF electrophoretic features and the slow migrating one that of HbC. CAE and HPLC can be used to characterise double heterozygosity.

Compound heterozygotes SC. Here, the main additional diagnostic problem is to differentiate the HbC (β6 lys) trait from some βS gene variants and other unrelated alleles.

a) some βS globin have a very slow electrophoretic mobility, similar to HbC, because a second mutation adds its own cationic charge to that of β6 val. Hb Harlem (β6 alan, 73 Asn) and Hb Ziguinchor (β6 val-58 Arg) are easily detected by CAE because their mobility is similar to that of Hbs and, hence, not like HbC. IEF and HPLC, here again, furnish valuable discriminative information.

b) others βS traits like HbE (26 Lys); HbE Saskatoon (22 Lys) and HbO Arab (121 Lys) are rather easily separated from HbC by the use of CAE and HPLC. Unlike S/E heterozygosity, the S/O Arab combination produces a severe SCD.
Phenotypic interferences

Homozygotes

1) Hb A2 and HbF levels. The Hb phenotype is stabilised at about 5 years old in patients with homozygous sickle cell disease (SCD) in their steady state. Hb A2 is inversely correlated to a gene number and HbF expression, so the values between 5% and 6% are commonly observed in SS patients homozygous for α+ thalassemia and having low HbF percentage. This latter phenotype overlaps significantly with that of S/β° thalassaemia compound heterozygosity which is common (about 30% SCD) in Mediterranean patients and less (5%) in west Africans and Caribbeans patients.

2) Atypical clinical severity in homozygous (SS) sickle cell patients. Along with other tests, the question of an electrophoretically and HPLC silent Hb variation of the α or βS globin must be studied. The simplest way to obtain such information is to study the Hb molecules with mass spectrometric methods and with globin gene sequencing. The suspected mutation can be localised in either α or β genes by PCR based methods such as SSCP or DGGE.

Other red cell genetic defects, such as enzyme or membrane disorders, may be involved in modulating the severity of the clinical expression of sickle cell disease.
Management of the haemoglobinopathies

DIMITRIS LOUKOPOULOS
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Conventional treatment

To this date, correction of the haemoglobinopathies at the level of DNA defects is not available. Conventional treatment is aimed at palliation of the patients’ complaints, thus ensuring an acceptable quality of life, and at preventing chronic and irreparable organ damage. Transfusion therapy has a place in both major haemoglobin disorders, i.e., thalassaemia major (TM) and sickle cell disease (SCD). The two conditions, the decision to initiate transfusion therapy must be made with caution because the procedure is not as innocuous as it may, at first sight, appear to be. Thus, the criteria for transfusion in the former group are mainly development of bone deformations and failure to grow, while in SCD the only indication is a high frequency of vaso-occlusive episodes, especially when these are associated with pregnancy or stroke. Unless it is very severe, anaemia per se is not a definite indication for transfusion therapy in SCD. Blood transfusions may be the route of transmission of several infectious agents and can cause massive deleterious deposits of iron in the parenchyma of organs, including the heart. With the careful regulations that presently govern blood administration, transmission of infectious agents (mainly HIV and relative viruses) appears to have significantly decreased; in addition, hepatitis B which has been a major concern in the past has now almost disappeared as a result of vaccination programmes. However, the problem of persistent hepatitis C remains unsolved for a considerable number of patients who will, sooner or later, develop liver cirrhosis and carcinoma.

Iron deposition becomes dangerous when the total serum ferritin levels exceed 4,000 µg/mL (corresponding to 5 mg/g of liver tissue) and ultimately leads to death, usually through heart or liver failure. Two main approaches are currently used to decrease the noxious effects of iron: the standard administration of desferrioxamine (DF, 40-60 mg/day over 5-7 d/week by subcutaneous infusion) and the recently established, but still controversial treatment with deferiprone, an orally active agent, which may cause excretion of considerable amounts of iron, albeit not achieving in all cases the desired iron balance. For the time being the question is still open. Several groups now administer deferiprone, alone or in combination with DF, to selected patients with TM, and are trying to establish the appropriate dosage and timing, its potential synergy with DF and, last but not least, its reported liver toxicity. Moreover, several alternative preparations and new drugs are currently under evaluation.

Symptomatic therapy and general assistance, including professional and social support in both TM and SCD are well standardised and need not be repeated here. It is fortunate that over the last years a large number of new drug analgesics have become available to combat painful sickle cell crises. Bone marrow transplantation (BMT) has cured several hundreds of patients with either TM or SCD. In carefully selected patients, i.e., in those who have not already develop serious liver damage, the probability of engraftment is more than 90%, while that of living without complications is more than 80%. Of course, BMT requires a fully histocompatible donor, which is not readily available these days, at least in the Western world, because most parents cease having children after they give birth to an affected one. However, the possibility of transplanting TM patients with cord blood cells which may adapt more easily to their environment than child or adult cells, opens a new therapeutic strategy, which definitely must be pursued. It is also of interest that establishment of mixed chimerism may prove beneficial; in thalassaemia, a persistent level of 30% donor cells is considered to be adequate to prevent chronic and irreversible organ damage.

Non-conventional treatment of the haemoglobinopathies

Novel treatments are targeted at various issues of the pathophysiology of the 6-thalassaemia. For the time being, none of the proposed approaches is fully effective or safe although most of them are based on sturdy data and arguments and merit all efforts and investments. Increasing the proportion (and amount) of foetal haemoglobin (HbF) is the most promising target. Other, more specific objectives are addressed below, separately for the TM and the sickle cell syndromes (Tables 1 and 2).
### Table 1. Potential targets for therapy of sickle cell disease.

<table>
<thead>
<tr>
<th>Prevention of the sickling process through artificial modification of the ( \beta \text{-S} )-chains</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Non-covalent agents</strong></td>
</tr>
<tr>
<td>Urea, aromatic acids, peptides. Of theoretical value; require unreachable concentrations</td>
</tr>
<tr>
<td><strong>Covalent agents</strong></td>
</tr>
<tr>
<td>Modification of the terminal amino acids</td>
</tr>
<tr>
<td>Cyanate, pyridoxal, tucaresol</td>
</tr>
<tr>
<td>Modification of the lateral amino acid chains</td>
</tr>
<tr>
<td>Glyceraldehyde, mustards etc</td>
</tr>
<tr>
<td>Modification of the 2,3 DPG binding site</td>
</tr>
<tr>
<td>Diaspirin and others</td>
</tr>
<tr>
<td>Nitrogen monoxide. Under evaluation</td>
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</tbody>
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<table>
<thead>
<tr>
<th>Inhibition of HbS polymerization through cellular hydration (lowering MCHC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDAVP, cetedil, monesin, tellurite, nystatin, chlorpromazine</td>
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</table>

<table>
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<tr>
<th>Inhibition of HbS polymerization through preventing cellular dehydration</th>
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<tbody>
<tr>
<td>Inhibition of calcium influx</td>
</tr>
<tr>
<td>Verapamil, diltiazem, nifedipine etc</td>
</tr>
<tr>
<td>Oral Mg pidolate; Mg aspartate</td>
</tr>
<tr>
<td>Inhibition of potassium efflux by inhibiting the Ca++ dependent K+ efflux (Gardos channel)</td>
</tr>
<tr>
<td>Clotrimazole, miconazole, charybdotoxin</td>
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<table>
<thead>
<tr>
<th>Prevention of intracellular oxidation and membrane damage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron chelators, vitamin E, acetyl-cysteine</td>
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</tbody>
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<table>
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<tr>
<th>Prevention of vaso-occlusion</th>
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<tbody>
<tr>
<td>Anticoagulant therapy</td>
</tr>
<tr>
<td>Vasoactive agents</td>
</tr>
<tr>
<td>Nitrogen monoxide, pentoxiphylline etc.</td>
</tr>
<tr>
<td>Inhibitors of cellular adhesion</td>
</tr>
<tr>
<td>Anti-integrine ( \alpha(\text{IIb}) )-anti-CD36, anti-VCAM1, anti-thrombospondine</td>
</tr>
<tr>
<td>By “coating” the red cells with polyethyleneglycol and other molecules</td>
</tr>
</tbody>
</table>

Beuzard et al (6); slightly modified.

### Table 2. Potential targets for the therapy of thalassaemia major. [Mostly theoretical up to now]

<table>
<thead>
<tr>
<th>Decreasing the excess of ( \alpha )-chains by</th>
</tr>
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<tbody>
<tr>
<td>• Interfering with the ( \alpha )-gene transcription</td>
</tr>
<tr>
<td>• Promoting synthesis of ( \gamma )-chains</td>
</tr>
<tr>
<td>• Increasing proteolytic degradation</td>
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</tbody>
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<table>
<thead>
<tr>
<th>Preventing intracellular oxidation and precipitation of the ( \alpha )-chain excess</th>
</tr>
</thead>
<tbody>
<tr>
<td>• By efficient anti-oxidative agents</td>
</tr>
<tr>
<td>Chelating free iron and free heme moieties thus preventing intracellular oxidations and membrane damage through neutralization of the active free oxygen radicals</td>
</tr>
<tr>
<td>Vitamin E, deferiprone, desferoxamine, acetyl-cysteine, quinolinic/indolinic nitroxide, dimethy-isoeugenol, N-allylsecoboldine, other</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Preventing premature death of the erythroid precursors and erythroblasts in the marrow</th>
</tr>
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<tbody>
<tr>
<td>• Anti-apoptotic agents</td>
</tr>
<tr>
<td>Bcl-XL, Heat Shock Proteins; other</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Preventing premature death of the erythrocytes in the peripheral blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>• By “coating” the red cells with polyethylene glycol or other substances, thus</td>
</tr>
<tr>
<td>• Masking the phosphatidyl-serine molecules of the membrane which serve as signals for the erythrophagocytosis of the red cells by the RES, or</td>
</tr>
<tr>
<td>• Inhibiting the binding of naturally occurring antibodies to the antigens of “senescence” RBC antigens, whose appearance on the RBC membrane is enhanced by the intracellular precipitation of the excess ( \alpha )-chains and the resulting oxidative damage.</td>
</tr>
<tr>
<td>Polyethylene glycol and other molecules</td>
</tr>
</tbody>
</table>

Beuzard et al (6); slightly modified.
Increase of foetal haemoglobin

Thalassaemic patients fare much better when they can complement the poor or null \( \beta \)-chain synthesis in their erythroid cells with \( \gamma \)-chains; this benefit is brought about not only through an increase in the total corpuscular haemoglobin content by the addition of varying amounts of HbF, but mainly through the neutralisation of deleterious effects of the \( \alpha \)-chains which are present in relative excess and precipitate intracellularly because of instability, causing premature death of the erythroid cells in the marrow (severe ineffective erythropoiesis) and the peripheral blood (haemolysis). On the other hand, it also well known that patients with various sickle cell syndromes have fewer or no painful crises when they can complement their HbS-containing erythrocytes with a varying amount of HbF. In fact, the latter prevents sickling by diluting the HbS molecules and by forming \( \alpha \beta \gamma \)-chain hybrids, which significantly inhibit polymer formation. These observations have been repeatedly confirmed by various experimental systems in the laboratory. Based on these observations, research towards understanding the mechanisms of the transition from foetal to adult haemoglobin synthesis, and possible reversion to foetal haemoglobin synthesis induced by pharmacological or other means have been pressing challenges for scientists active in this field. There are two main pathways currently moving towards these goals: (1) selective proliferation of erythroid cells containing increased amounts of HbF and (2) activation of the \( \gamma \) genes across all the patient’s erythroid precursors.

Selective proliferation of erythroid cells containing increased amounts of HbF. A major advance in this process was the observation of increased numbers of F-red cells in patients with aplastic anaemia during their recovery phase, in patients with leukaemia on entering remission, and in patients recovering after a successful bone marrow transplant. Since the common denominator of all these situations was acute bone marrow expansion after a phase of severe suppression, the concept of recruitment of early cell progenitors which retain the programme of \( \gamma \)-chain synthesis but do not proliferate in the normal marrow unless acutely called up was gradually established. Cytostatic drugs appear to act through this mechanism. Of these, hydroxyurea (HU), a potent ribonucleotide reductase inhibitor has gained wide acceptance because it has proven effective and was well tolerated with no major side effects.

Following the animal experiments of Letvin et al., several medical groups in the USA have administered HU to Black American patients with sickle cell disease in an attempt to increase Hb. The reported results showed an unequivocal increase of HbF but were not controlled enough with regards to clinical efficacy. The clinical efficacy was clearly shown in 1995, when a multicentre double blind study of 205 Black American patients with SCD were treated with HU and compared to an equal number of patients given an appropriate placebo revealed a statistically significant decrease of the frequency of yearly crises and need for transfusions along with an equally significant increase of the interval to the next crisis. In keeping with the earlier observations HbF levels had also significantly increased. The patients were homozygous sicklers and were treated in various sickle cell centres all over the United States. Hydroxyurea has proved also effective in Caucasian compound heterozygotes for HbS and severe \( \beta \)-thalassaemia. This was first reported by the author’s group in 1995 and since been updated. There are now following more than 70 patients, some of whom have been on HU for more than 9 years and have shown an impressive response with regards to both decreased frequency and severity of crises and increased HbF. Meanwhile, several similar reports from other European countries where the problem of sickle cell disease is equally pressing because of migration have confirmed the beneficial results of HU not only in adults but also in children. According to present concepts, the benefits of HU derive not only from the increase of HbF but also from the decrease of white blood cells (WBC) and potential blood cell-endothelial cell interactions. As a rule, tolerance of HU as well as immediate toxicity (decrease of platelets and WBC and skin changes) are quite acceptable. However, the question of long-term toxicity, particularly leukaemogenesis remains open. Selective proliferation of \( \gamma \)-thalassaemia intermedia for experimental rather than practical reasons. A relatively large group of such studies has recently been reported by our group. A summary of the results is following a few weeks of HU, HbF but not total haemoglobin levels increased significantly in all patients; along with these changes, transferrin receptors decreased considerably and the patients declared feeling better. Our interpretation is that these results indicate a decrease of ineffective erythropoiesis and hence, a lesser wastage of energy along with an improvement of life quality. The question is still open. Administration of recombinant erythropoietin, alone or in combination with HU, has also been repeatedly reported in recent literature; results show an unequivocal increase of total haemoglobin as well as of HbF, but the cost of r-huEPO and the danger of inducing undesirable bone marrow expansion, makes this approach impractical.

A divagation of the \( \gamma \) genes across all erythroid precursors in sickle cell or thalassaemic patients. The most representative agents with this potential are 5’ azacytidine and butyric acid with its analogues. 5’ azacytidine, a potent cytostatic drug which is considered to act through inhibition of methylation of the \( \gamma \)-Chain genes has, in fact, given good proof of its efficiency but is potentially highly carcinogenic and cannot be
advocated for clinical use. Butyric acid (BA), a relatively non-toxic agent, with a high potential for the induction of α-chain synthesis in vitro, has, however, repeatedly been evaluated in various clinical trials with controversial albeit promising results. BA is thought to act at the level of DNA by (a) promoting α-mRNA transcription and (b) inhibiting the histone acetylase, thus preventing dense packaging of transcription and (b) inhibiting the histone acetylase, making it a feasible with various products. Of particular interest is the observation that oral administration of BA is generally well tolerated.

Increased α-chain transcription is not confined to BA since other short chain fatty acids have similar effects. This observation set off a large search among already available drugs whose molecules contain fatty acid groups which might enhance HbF synthesis. Thousands of other products are currently being evaluated by large scale robotic systems. The clinical results are still conflicting. The initial very satisfactory results of Perrine et al. were not fully confirmed in subsequent studies. However, in a recent well controlled trial, Atweh et al reported a significant increase in both total haemoglobin and HbF in 9 of 11 patients with homozygous HbS disease who were given four day infusions of arginine butyrate followed by drug free intervals of 10-24 days over several months. Ongoing research is aimed at finding substances which may have the above effects after oral administration, for example valproic acid, isobutyramide, sodium phenylbutyrate and other still more potent agents.

Can we enhance the oxygen release of HbF? This is a question which has not yet been widely explored but is very pertinent because the oxygen affinity (OEC) of HbF is higher than that of HbA. Shifting the haemoglobin oxygen equilibrium curve to the right is now feasible with various synthetic allosteric modulators. Of these, the bezafibrate derivative RSR-4, which was recently evaluated in vitro, is certainly promising at the level of concentration of 250 µM shifted: the OEC of a blood sample containing nothing but HbF from 18.7 to 26.5 mmHg; the latter can be easily reached in experimental animals without any side effects. Doubling the concentration resulted in further increase of the p50 of the sample to 37.3 mmHg. Other agents potentially useful in SCD

1. drugs capable of increasing the oxygen affinity of the HbS molecule by forming covalent bonds with aminocarboxylates which are crucial for the transport of O2 (for example: 3,5-dibromosalicyl fumarate which inhibits the binding of 2,3-DPG and tucarosol, which increases the oxygen affinity of HbS considerably by binding to the N-terminal end of the α-chains);
2. nitric oxide (NO) which may increase the O2 affinity of HbS (although its primary action is significant vasodilatation);
3. magnesium (pitoliate or aspartate) which may prevent K+ loss and cellular dehydration through inhibition of the Mg dependent K+Cl− co-transport system. In fact, there are now adequate data showing that oral administration of tolerable amounts of the above Mg salts in patients with sickle cell disease results in disappearance of dense cells and decrease of red cell heterogeneity; related clinical data are not yet available, K+ loss may also be prevented by inhibiting Ca++ dependent K+ efflux (Gardos channel) by clotrimazole or other antifungal agents;
4. agents which block the Ca++ influx into the red blood cells, e.g. nifedipine, have a theoretical application, but no strong clinical support;
5. antioxidants (vitamin E, aminocysteine, amifostine?) prevent membrane oxidation and damage caused by the abundant release of free oxygen radicals;
6. anticoagulant therapy preventing vaso-occlusion by inhibiting any kind of local coagulation; to be explored: low dose heparin, agents blocking the interaction of fibrinogen and platelets with the GpIIb/IIIa and GpIb platelet proteins;
7. vasoactive agents (nitric oxide, pentoxyphilline, etc.) bringing about ample vasodilatation;
8. inhibitors of the adhesion of the sickled red cell onto the endothelium (ReothRx, a general ionic surfactant, hydroxyurea resulting in decreased expression of the integrin αβ and the antigen CD36 on the cellular surface, anti-CD36, anti-αβ, anti-VCAM 1, anti-thrombospondin, etc.;
9. polyethylene glycol and other agents coating the red cells in an attempt to make them less recognizable by the macrophages of the RES, thus increasing their survival in the peripheral blood.

Other approaches potentially useful in thalassaemia major

These are listed in Table 2, but, apart from the promotion of α-chain synthesis or the selective proliferation of cells capable of synthesising HbF, all others are rather theoretical and have not yet been experimentally evaluated. At present, the possibility of reducing intracellular oxidative processes resulting from α-chain precipitation and the presence of free iron appears more accessible and is already attracting strong experimental effort.

References
Cyclin-dependent kinases and their inhibitors in haematological malignancies

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Disregulation of the cell cycle machinery leads to uncontrolled and unwanted cell division, which is a key event in the development of cancer. Repeatedly, the tumour suppressor genes TP53 and RB have been shown to influence the cell cycle. Also, ever since one of the cyclin-dependent kinase inhibitors (CDKIs), p16INK4a, was found to be altered in transformed cell lines and primary cancers, CDKIs have been viewed as candidate tumour suppressor genes. We will briefly review the alterations of the components of the cell cycle machinery in haematological malignancies.

Cell division is governed by a concerted action of a set of homologous serine/threonine kinases known as cyclin-dependent kinases (Cdks), and their regulatory subunit, cyclins. Cdks have to be activated and inactivated in an intricate manner at specific time points during the cell cycle, and this is facilitated by the accumulation and degradation of phase-specific cyclin molecules (Figure 1).

Cyclin D, cyclin E and their associated Cdks have a critical function in the G1/S progression of mammalian cells.1 D-type cyclins (cyclin D1, 2 and 3) are associated with either Cdk4 or Cdk6, which means that six combinations with possibly distinct functions can occur. The combination appears to be dependent on cell lineage. D-type cyclins are unique in that they are induced by growth factors and maintained as long as the factor is present, which means that this group of cyclins is one of the links between external signals and regulation of the cell cycle. Another G1 cyclin, cyclin A, accumulates slightly later in the G1 phase than cyclin D1 and is associated with Cdk2. The kinase activities of both the cyclin D/Cdk4/6 and cyclin E/Cdk2 complexes are believed to be necessary for the G1/S transition. As cells enter the S phase, the cyclin E protein level and the associated kinase activity gradually diminish. Activity of G1/S cyclin/Cdks is also counteracted by their cytoplasmic translocation, and by CDKIs, as described below.

These G1-Cdks are believed to phosphorylate and thereby inactivate pRB, which is the protein product of the retinoblastoma gene (RB), the first described tumour suppressor gene. The phosphorylation status of pRB is tightly regulated during the cell cycle. During the G1 phase, pRB exists in an un- or underphosphorylated form, whereas in late G1 through to the end of the M phase, it is extensively phosphorylated. It then undergoes rapid dephosphorylation during anaphase. The growth inhibitory effect of pRB is predominantly associated with its underphosphorylated form, which binds to a family of cellular transcription factors known as E2F.2 The binding to pRB switches E2F from a transcriptional activator to a repressor. Many genes important for cell proliferation have an E2F binding site in their promoter, and therefore might be activated to be expressed at the G1/S boundary by E2F freed from the hyperphosphorylated form of pRB. pRB also recruits histone deacetylase to repress E2F-responsive genes directly.3

Expression of cyclin E is also induced by E2F, which means these two molecules form a positive feedback loop. Thus, the decision point for a cell to enter the S phase (R point) might be the moment when E2F activity induced by cyclin D/Cdk4/6 is just robust enough to fire the autonomic increase of cyclin E and E2F activities.4

Another important checkpoint, the mitotic checkpoint, exists in the cell cycle to prevent aneuploidy, which is often observed in cancer cells. During cell division, sister chromatids are attached, via an apparatus called kinetochores, to microtubules which are cast from two opposing mitotic spindles, to make sure that each of the paired chromatids is divided evenly into two daughter cells. Failure of this step triggers the mitotic checkpoint, which halts the cell cycle and provides the cell with an opportunity to repair the abnormality. Recently, a gene encoding one of the key molecules in the mitotic checkpoint, hUB1, was shown to be mutated in some colorectal cancers.5 Perturbation of this checkpoint may be responsible for other types of cancers that show aneuploidy.

The tumour suppressor p53 has recently been found to have homologues, p73 and p51.6–7 Although these homologues bind to and activate the same DNA sequence as p53, the stimuli that induce these genes have not yet been defined. Unlike TP53, which is mutated in a variety of cancers, alterations of these homologues appear to be quite rare. Thus, we do not know yet whether these p53 homologues are implicated in carcinogenesis. We have found that p73 is unexpressed and the gene is highly methylated in 30% of samples from patients with acute lymphocytic leukaemia and B-non-Hodgkin’s lymphoma samples (Kawano S, et al. Blood 2000; in press).

Cyclins in cancer

Several lines of evidence link aberration of the cell
cycle to the development of cancer (Table 1). Cyclin D1/PRAD1/bcl1 gene is located on chromosome 11q13, and is sometimes overexpressed in cancers by several mechanisms. The gene is frequently translocated to chromosome 14, (t(11;14)(q13;q32)) in mantle cell lymphomas as well as centrocytic lymphomas and intermediately differentiated lymphocytic lymphomas (the latter two may occasionally be mantle cell lymphomas). Presumably, the cyclin D gene locus is translocated adjacent to the immunoglobulin heavy chain gene locus at 14q32 and is activated by the regulatory element of the latter, which is efficiently expressed in B-lineage cells. In some B-cell tumours and breast cancers the cyclin D1 gene is overexpressed in the absence of chromosomal translocation. The gene is often rearranged to the parathyroid hormone gene locus in benign parathyroid adenomas, and either amplified or overexpressed in several types of cancers including breast cancers and head and neck cancers. Individuals with head and neck cancers whose initial tumours had overexpression of cyclin D1 were more likely to have a recurrence.

The overexpression of cyclin E has been reported in several types of cancers including chronic lymphocytic leukaemia. In breast cancers, the degree of overexpression of the cyclin E protein tended to parallel the aggressiveness of the disease.

We recently cloned human cyclin A1. The function of this cyclin is unclear. It is highly expressed dur-
ing murine spermatogenesis and male mice that have deletion of cyclin A1 are infertile. We and others have found cyclin A1 to be highly expressed in about 25% of acute myeloid leukaemia samples. The significance of this prominent expression is unclear.

Cyclin-dependent kinase inhibitors
Cyclin-dependent kinase inhibitors (CDKIs) are a class of small proteins that inhibit the activity of Cdks. These proteins are important for the controlled progression of the cell cycle, and are therefore candidate tumour suppressor genes. Mammalian CDKIs can be classified into the Kip/Cip family and the INK4 family.

Kip/Cip family
This family comprises three members, p21WAF1/CIP1, p27KIP1 and p57KIP2, which have homology in their N-termini and inhibit various types of cyclin/Cdk complexes. p21 is induced by the wild type but not the mutant p53 gene. For example, γ-irradiation induces p53, which increases the transcription of p21; increased level of this CDKI inhibits cyclin E/Cdk2 kinase, thereby causing a G1 arrest of the irradiated cells. Moreover, the p21 protein can inhibit DNA replication by binding directly to PCNA and thereby inhibiting the ability of the latter to activate DNA polymerase. Thus, the p21 protein appears to mediate the cell cycle arrest caused by DNA damage in both G1 and S phases via distinct pathways.

However, the activation and activities of p21 are more complex than portrayed above. p21 can be induced as an immediate early gene by certain differentiating reagents even in the absence of p53. Furthermore, p21 has also been implicated in protecting differentiating cells from apoptosis.

We have studied genomic DNAs from 351 primary samples from 14 types of malignancies and 36 cancer cell lines of various types. We did not find any alterations which would result in inactivation of the p21 gene. A case of Burkitt's lymphoma with hemizygous mutation of p21 has been reported. Genetic changes that cause complete loss of p21 function may be fatal for cells.

p27KIP1 protein increases in response to external signals that induce cells to exit the cell cycle. For example, resting T-lymphocytes begin to synthesize Cdks and cyclins in response to T-cell receptor-mediated stimuli, but they cannot enter the cell cycle until the second mitogenic signal by IL-2 leads to down-regulation of p27. Thus, the level of p27 protein provides a threshold for the G1 cyclin/Cdk complexes to overcome. Its expression is independent of the status of p53.

Our extensive analyses of the p27 gene in cancers and cell lines found only several cases of an altered p27 gene in adult T-cell leukaemias. Overall, alteration of this gene is very rare. However, immunohistochemical studies have shown that in certain lymphomas, decreased level of p27 was correlated with a high proliferative rate as measured by Ki-67 expression. Also, lymphomas with a low level of p27, either alone or in combination with high levels of cyclin E26, were associated with a poor prognosis in several solid cancers, low level or lack of p27 expression correlated with tumour progression, poor prognosis and sometimes metastasis. Besides transcription, important regulation of protein level of p27 occurs through the regulation of its rate of translation, and rate of degradation by the ubiquitin-proteosome pathway.

The expression of the p57 CDKI gene is also independent of p53, and is abundant in terminally differentiated cells. The gene is on chromosome 11p, where the expression of several genes is known to be controlled by imprinting. The locus for the Beckwith-Wiedemann syndrome has been mapped to this region, and is also imprinted. p57KIP2 knock-out mice exhibit phenotypes also seen in patients with Beckwith-Wiedemann syndrome. It has therefore, been suggested that the p57KIP2 gene is involved in the development of this disease, although the precise
mechanism of its inactivation is unclear. The level of p57, like p27, appears to be critical for a cell to exit the cell cycle. However, p57 has not been implicated as having a causal role in cancer.

INK4 family

The members of the INK4 family include p16INK4a, p15INK4b, p18INK4c and p19INK4d, which all have ankyrin repeats as a common structure. Unlike the members of the Kip/Cip family, which form complexes with cyclin/Cdk, the INK4 family of proteins forms binary complexes specifically with Cdk4 and Cdk6, and inhibits their activities. When synchronised human lung fibroblasts are stimulated with serum, the p16 protein gradually increases to a maximum at the G1/S boundary. This suggests that it might be responsible for inactivating cyclin D/Cdk4/6 at the G1/S boundary when pRb has been adequately phosphorylated to allow the cells to enter the S phase. In cells driven to premature senescence by constitutive expression of oncogenic ras, accumulation of p16 and p53 is critical for their growth arrest.

Surprisingly, the INK4a locus codes for another, unrelated protein, p14ARF (p19ARF in mice), utilising an alternative reading frame. INK4a knock-out mice lacking both the p16INK4a and p19ARF genes develop spontaneous cancer early in their life, and fibroblasts derived from these mice can be transformed by oncogenic ras alone. However, these phenomena can be largely reproduced by selective disruption of p14ARF.

The p14 protein binds to and promotes the degradation of the MDM2 protein, which itself inactivates p53. This is a very powerful mechanism of inactivation of the p53 protein, p14ARF, utilising an alternative reading frame.

INK4b

INK4b is highly homologous to p16INK4a, but distinct from p16 in that it is increased in the setting of TGF-β-induced cell cycle arrest; p15INK4b may be a mediator of TGF-β signals to the cell cycle control. The INK4b locus lies only 25 kilobases from the INK4a locus, and the two loci are often simultaneously deleted. Therefore, we do not know which of these two genes is the target for inactivation during tumorigenesis. Hemizygous deletions of INK4a/b with intact remaining alleles are also sporadically observed in certain types of cancers with frequent homozygous deletions of these genes. Because CDKIs exert their regulatory effects on the cell cycle by means of subtle quantitative changes, such hemizygous deletions, which may lead to a decrease of their gene products, might be functionally significant.

In cancers without genetic alteration of INK4a/b, the protein products may be absent for epigenetic reasons. The promoter regions of the INK4a/b genes are GC-rich and contain many CpG dinucleotides, which are recognised by methyltransferase. Resultant hypermethylation of such CpG islands often leads to transcriptional silencing of the gene. Both the INK4a and INK4b are frequently inactivated by hypermethylation in B- and T-cell lymphomas, Burkitt’s lymphomas and multiple myelomas. In myeloid leukaemias in which genetic alteration of INK4a/b is infrequent, INK4b seems to be selectively hypermethylated in myelodysplastic syndrome, INK4b hypermethylation has been associated with bony marrow involvement. Further studies are required to determine the significance of INK4a/b gene silencing by hypermethylation; only a limited number of studies have analysed actual levels of the INK4a/b mRNA or protein.

Alteration of INK4c and INK4d, by either deletion or hypermethylation, seems to be very rare in cancers.

Conclusions

A growing body of evidence strongly suggests that the inactivation of CDKIs, either by a genetic or epigenetic mechanism, is pivotal for tumorigenesis. Future challenges include determining how extracellular information is transmitted intracellularly for control of the cell cycle, and identifying other substrates of Cdkks in vivo. In addition, further exploration of mitotic checkpoint genes and cell cycle-related phosphatases may provide insights into the pathogenesis of selected haematopoietic malignancies.

Acknowledgements

We thank our colleagues for sharing their data and for helpful discussion, and Ms. Kim Burghin and Sayuri Nakayama for their secretarial help.

References


46. Zhang S, Ramsay ES, Mock BA. Cdkn2a, the cyclin-dependent kinase inhibitor encoding p16INK4a and p19ARF, is a candidate for the plasmacytoma susceptibility locus, Pctr1. Proc Natl Acad Sci U S A 1998; 95:2429-34.


Initiation of DNA replication in normal and neoplastic cells

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The initiation of DNA replication is a crucial and inescapable stage of cell proliferation. Entry into S-phase is the purpose and point of convergence of many signalling pathways.

In this article I shall review the initiation of DNA replication and its control and shall then consider the extraordinary potential value of antibodies raised against the proteins that regulate DNA replication as diagnostic reagents for improving the early diagnosis of cancer. I shall argue that some of the proteins that regulate DNA replication may serve as markers for the early dysplastic changes that precede malignancy.

The mechanism of initiation of DNA replication and the control of initiation are highly conserved from yeasts to vertebrates including humans. Initiation involves step-wise assembly of multi-protein pre-replication complexes at sites where replication will initiate. Pre-replication complexes in turn recruit proteins of the replication fork responsible for enzymatic synthesis of DNA.

The large genomes of eukaryotic cells pose a particular problem, requiring initiation at many sites on each chromosome. If initiation occurred only at one site, each human chromosome would take more than one month to complete replication. Multiple initiations pose a problem of co-ordination to ensure that all of the DNA is fully replicated but that none of it replicates twice within a single cycle. Replication of 99.999% of the genome is not enough because the unreplicated fraction would cause chromosome breakage at cell division. A further problem arises from the fact that all of the DNA must be copied once and only once, without re-initiation in a single cell cycle. I shall review below how this is achieved by sequential assembly and disassembly of pre-replication complexes which serve as a ratchet to co-ordinate multiple initiations and to prevent re-initiation from occurring within a single cycle.

The pre-replication complex couples DNA replication to the cell cycle

Coupling DNA replication to the cell cycle involves two problems. The first is controlling the time and place of DNA replication, ensuring that proliferation is restricted so that cells behave as integrated communities and only proliferate in response to appropriate signals. Proliferation in the absence of appropriate signals, or autocrine provision of the signal by the cell itself, can result in the anti-social behaviour of the cancer cell. The second problem is that of preventing re-initiation of replication between consecutive mitoses, which would result in local gene amplification. Both of these aspects have been studied intensely recently and have resulted in a remarkable convergence of genetic studies in yeasts with cell biology and biochemical studies in Xenopus, Drosophila and humans. These various studies have identified the same family of proteins that bind sequentially to DNA to license unreplicated DNA for one round of replication. The proteins concerned are summarised in Table 1 and Figure 1. The origin recognition complex (ORC) consists of six protein subunits that bind to the sites at which replication will initiate. ORC is known to bind to specific DNA sequences in the yeast S. cerevisiae, but the specificity of its binding in higher eukaryotes remains unresolved. Although the ORC is required for initiation of replication in Xenopus embryos, these embryos appear to initiate DNA replication at sites that are not specified by primary DNA sequence. ORC serves as a landing pad for binding of Cdc6, a single subunit protein that shows homology to some ORC subunits, such as ORC1. Cdc6 in turn serves as a landing pad for another six subunit complex, the mini-chromosome maintenance or MCM complex, consisting of six conserved subunits that show DNA helicase activity. Although loading of MCMs onto the DNA template is dependent on Cdc6 and ORC, the amount of MCMs loaded appears to exceed the amount of Cdc6 or ORC by a factor of 10-20 fold. As replication proceeds, MCMs are displaced from the template so that replicated DNA lacks MCMs but unreplicated DNA is marked by their presence. In this way un-replicated DNA can be distinguished from replicated DNA enabling the cell to prevent re-initiation of replication within a single cell cycle.

In addition to the proteins listed above, other proteins also form part of the pre-replication complex, namely Cdc45, Cdc7 and Dbf4. The partnership of Cdc7 and Dbf4 closely resembles that of a cyclin dependent kinase (Cdc7) and its cyclin partner (Dbf4) and this complex is known to be capable of phosphorylating some of the replication proteins.

Quiescent cells lack components of the pre-replication complex

When cells opt out of the proliferation cycle and become quiescent, they rapidly destroy Cdc6, but retain ORC bound to their chromatin. In addition, they displace MCMs from the chromatin so that they remain in the nucleoplasm but not bound to chromatin. MCMs are then progressively degraded so
Table 1. Proteins of the pre-replication complex.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>ORC (six subunits)</td>
<td>Binds sites of initiation and recruits Cdc6</td>
</tr>
<tr>
<td>Cdc6 (also = cdc18 in S. pombe) (one subunit)</td>
<td>Binds to ORC on DNA and and recruits MCMs</td>
</tr>
<tr>
<td>MCMs (Minichromosome maintenance complex (six subunits)</td>
<td>Binds to Cdc6/ORC; DNA complex in 10-20 fold excess. Marks unreplicated DNA, DNA helicase.</td>
</tr>
<tr>
<td>Cdc7</td>
<td>A 'cyclin' dependent kinase</td>
</tr>
<tr>
<td>Dbf4</td>
<td>Cyclin-like partner of Cdc7</td>
</tr>
</tbody>
</table>

Replication competent chromatin

Proteins of the pre-replication complex mark unreplicated DNA and license it for a single round of DNA replication.

The Origin Recognition Complex (ORC) is bound throughout the cell cycle. ORC binding allows the binding of Cdc6 that, in turn, allows binding of the Minichromosome Maintenance (MCM) complex in excess. The MCM complex marks unreplicated DNA, and it is displaced by DNA replication, distinguishing replicated DNA from unreplicated DNA. The figure was kindly provided by A. D. Mills.
these to investigate the regulation of human cell proliferation.

Proteins of the pre-replication complex are valuable markers of cell proliferation.

Following the observation that some proteins of the pre-replication complex, namely Cdc6 and MCMs, become degraded during quiescence, we have investigated the value of antibodies raised against these proteins as markers of proliferating cells in histological and cytological samples. As outlined below, these antibodies show exceptional promise as markers of dysplasia and neoplasia. Not only can they detect dysplastic or neoplastic cells in biopsies but they are also valuable reagents for detecting exfoliated cells in body fluids as indicators of the presence of carcinomas. Furthermore, they also show potential value for differential diagnosis and estimation of prognosis for a range of neoplasias. Antibodies against Cdc6 or MCM proteins both give good results but we have focused on the use of MCM proteins, rather than Cdc6 on the grounds that MCMs are more abundant antigens than Cdc6 (see above) and Cdc6 is a substrate for regulated degradation in the cell cycle, meaning that it is a less robust antigen than MCMs.

Diagnosis of dysplasia and neoplasia using anti-MCM antibodies

A. Immunoenhanced cervical smears can decrease false negatives.8 We have assessed the value of MCM antibodies for detecting dysplastic and neoplastic cells in cervical smears. Recognition of abnormal cells in smears is notoriously difficult. We have found that it is possible to superimpose staining for MCM antibodies over a light Papanicolaou stain so that dysplastic cells can be recognised by the presence of MCM antibodies which are completely absent from both normal endocervical and ectocervical cells. Preliminary blinded trials of patients presenting at colposcopy clinics have succeeded in identifying cases of cervical neoplasia which had not been detected by routine Papanicolaou staining, but which have since been confirmed.

B. General detection of dysplasia and neoplasia in biopsies.7 Following the encouraging results obtained using anti-MCM antibodies on cervical smears, we have investigated their value as markers of dysplasia and neoplasia in biopsies from a wide range of tissue sites. Whereas MCM expression is detected in only proliferating cells of normal epithelia, they are expressed in the great majority of cells of dysplastic and malignant tissues. Clear differences were found between normal and neoplastic tissues for cervix, skin, lung, oesophagus, colon, bladder, prostate, kidney, ovary and endometrium.

C. An immunoassay for bladder cancer by detection of MCM antigens in urine.9 In addition to histological and cytological analysis using antibodies against MCM proteins, we have also developed an immunoassay for urothelial cancers that detects MCM proteins in urine. The basis of the method is that normal cells exfoliated into urine have ceased expressing MCM proteins before they are exfoliated. However, this is not true of transitional cell carcinomas which shed MCM positive cells into the urine. We are currently extending trials of this method as well as adapting it to assays of other types of carcinoma using other body fluids.

D. Towards antigenic screens for the common carcinomas.

The methods outlined above offer the possibility of earlier detection of the common carcinomas by assaying body fluids for either exfoliated cells expressing MCM antigens, or soluble antigen detected after the cells have been dissolved. If these trials prove successful then it may be possible to establish screens for other common carcinomas along the lines of the present cervical smear test for cancer of the cervix.

It is important to note that the proportion of dysplastic or neoplastic cells that stain with antibodies raised against MCM proteins is higher than that observed for known markers of cell proliferation, such as PCNA or Ki67. Furthermore, it is important to note the rationale for choosing these proteins as markers. Thus, there are many parallel pathways of signal transduction from growth factors through membrane receptors and intracellular signal transduction mechanisms that trigger cell proliferation via the protocarcinogenic pathways. However, as explained above, the mechanism of initiation of DNA replication is highly conserved amongst all eukaryotic organisms examined so far. Thus, in order to proliferate it is essential for cells to express MCM proteins, making these valuable markers for clinical exploitation.

An alternative simple method for detecting S-phase cells in tissue biopsies9

Antibodies against MCM proteins detect cells in all stages of the proliferative cell cycle, including G1S and G2 phases, but not G0. The value of these markers arises from the absence of MCM proteins from the great majority of differentiated cells in the body but their presence in the great majority of tumour cells.

We have recently described an alternative approach for rapid detection of a subset of proliferating cells, namely only those that are in the S-phase of the cell cycle. The method is extremely simple and based on the protocol of our standard control reactions for the cell-free DNA synthesis systems we have developed. Thus to exclude any contribution from contaminating S-phase nuclei in the G1 nuclear template preparations, we routinely assay duplicate reactions but replacing S-phase cytosol by a simple buffer containing deoxyribonucleoside triphosphates and ribonucleoside triphosphates, S-phase nuclei elongate in this buffer as well as in S-phase cytosol, whereas G1 nuclei will only initiate in S-phase cytosol and not buffer. Since we routinely store template nuclear preparations in liquid nitrogen, we argued that it should be equally possible to incubate tissue sections from frozen biopsies in a similar buffer and to observe incorporation of fluorescently tagged pre-cursors into only S-phase nuclei.

We found that incubations as short as 15 minutes allow S-phase nuclei to be detected in routine tissue
biopsies. Although it has been possible to detect S-phase cells before, this has involved use of radioactive or ligand tagged nucleotides \textit{in vivo}. The method described in reference \#9 completely bypasses the need for incubation \textit{in vivo} but exploits run-on replication \textit{in vitro} to achieve the same ends.

Conclusions

Studies of the control of DNA replication in eukaryotic cells have identified a family of proteins that are essential for initiation of DNA replication in all types of eukaryotic cells studied so far. The presence of these proteins can be used to detect cells at any point in the proliferative cell cycle, but not in quiescence. Antibodies against MCM proteins are expressed in only a small minority of cells in normal tissues, namely those cells that are undergoing proliferative cycles. In contrast, anti MCM antibodies stain most cells in malignant or pre-malignant lesions. The detection of MCM antigens in exfoliated cells from body fluids offers an opportunity to screen for common carcinomas at stages which would be undetectable by other methods.

In addition to the value of anti-MCM antibodies as markers of cells that are locked in the proliferation cycle, rather than quiescence, a simple adaptation of cell-free replication systems has made it possible to detect S-phase cells in normal tissue biopsies by running a DNA replication elongation assay on frozen sections of tissue biopsies. This technique can provide further information about the fraction of cells in a tumour that are actively synthesising DNA, giving prognostic as well as diagnostic information.

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References

Exploiting the p53 pathway for cancer diagnosis and therapy

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The human p53 gene encodes a 393 amino acid nuclear phosphoprotein that functions as a tetrameric nuclear transcription factor to activate genes involved in growth arrest and apoptosis in response to DNA strand breaks and other cellular stresses. Studies with p53 knock-out animals have shown that this pathway is a key component of the normal apoptotic response to ionising radiation and in the absence of p53 animals become very prone to the development of neoplasia. The p53 gene plays a critical role as guardian of the genome in preventing human neoplasia. Individuals inheriting only a single active allele of the gene are very tumour prone (the Li-Fraumeni syndrome). Perhaps most striking is the high frequency of p53 mutations that occur somatically. More than 2,000 different p53 mutations have been discovered and more than half of human tumours completely lack functional p53 protein. This allowed us and others to demonstrate the vast range of tumours that use p53 as determined by immunohistochemical analysis of archival fixed tissue samples using new antibodies to p53 that worked on formalin-fixed sections.

The levels of p53 protein are very low in normal cells because the protein has a very short half-life. In tissue culture systems p53 protein levels rise in normal cells exposed to a variety of DNA damaging agents. Here we analyse this response in vivo and find extremely tight control of the cell and tissue type specificity of the response. While studying the interaction of p53 with a negative regulatory protein mdm2 we developed a novel "mini-protein" that bound mdm2 tightly, mimicking p53. We discovered that blocking the mdm2 p53 interaction leads to the stabilisation of p53, this implies that the mdm2 protein normally targets p53 for degradation. It is presumably this mechanism that is blocked in cells exposed to DNA damage. These findings are discussed in the context of novel approaches to the development of anti-cancer chemotherapy.

Results

Regulation of the p53 response in vivo

We wanted to determine whether the accumulation of p53 protein in cells exposed to DNA damage seen in tissue culture models also occurred in vivo in response to physiological levels of DNA damage. Dr Peter Hall exposed a patch of his skin to a physiological dose of solar mimetic UV light sufficient to induce a mild erythema. Samples of skin taken before and after irradiation showed a clear and dramatic induction of p53 in the nuclei of epidermal and dermal cells following UV irradiation. An extensive follow-up study in mice depended on the generation of a new polyclonal antibody to murine p53 suitable for use on fixed tissue sections. With this new reagent (CM5) it was possible to see p53 accumulation in response to whole body irradiation. An initial survey showed clear p53 accumulation and apoptosis in the classically radiation-sensitive tissues, for example spleen, thymus, gut and hair follicles. In other tissues p53 accumulated but there was no obvious evidence of apoptosis; for example, this pattern was seen in the osteocytes of the bone. Finally in some tissues no accumulation of p53 at all could be detected using this simple immunohistochemical cut-off of sensitivity.

The response in the intestine

A more detailed investigation of the radiation response in the small and large intestine revealed that p53 accumulation and radiation-induced apoptosis occurred in this tissue 4 hours after exposure to 5Gy of whole body irradiation. The pattern of expression of p53 as determined by immunohistochemistry with CM5 was very restricted. In the small intestine only a thin layer of cells showed a clear apoptotic response to radiation and it was in this layer of cells that p53 staining was apparent. The positional correlation between radiation-induced apoptosis and radiation-induced p53 accumulation was very striking. To prove that the apoptosis was p53-dependent we repeated the study using p53 knock-out mice. The results showed clearly that this early (4h) apoptotic response to radiation was completely absent in the p53 knock-out strain but clearly present in p53 wild type and heterozygote littersmates. Thus the p53 gene is essential for the normal physiological response to radiation and its restricted expression correlates precisely with the restriction of the biological response. Since all cells in the tissue presumably received the same dose of radiation these results suggest that the p53 response in vivo is extremely tightly regulated. The exact level at which this regulation operates is not yet clear. It could reflect difference in
the persistence time of p53 inducing DNA lesions (i.e. reflected in differential repair rates) since agents that delay repair of ionising radiation-induced DNA damage (e.g. PARP inhibitors) have been shown to prolong the p53 response in tissue culture systems. Alternatively it could reflect differential sensitivity of the signal transduction pathways that respond to DNA damage by preventing the degradation of p53.

Development of p53 reporter gene transgenic mice

The fact that in some tissue p53 accumulation was not accompanied by apoptosis led us to speculate whether this was because the accumulated p53 was inactive. There have been several reports of cell types in culture which accumulate transcriptionally inactive forms of wild type p53. To address this issue we constructed transgenic mouse strains in which a p53 responsive promoter and the RGC p53 response element to drive β-galactosidase expression. In tissue culture systems we have been able to develop cell lines containing integrated copies of this reporter gene that show clear damage induced p53 dependent reporter gene expression and have an undetectable level of reporter gene expression in the absence of DNA damage. Two independent strains of these p53 reporter mice were successfully bred and analysed for the radiation responsiveness of the reporter gene expression. The results, discussed in depth elsewhere, confirmed that the induced p53 was transcriptionally active in most tissues types. The authors also again highlighted the extreme cell and tissue type restriction of p53 dependent gene activation in response to DNA damage in vivo.

Regulation of the p53 response by Mdm2

The challenge raised by the recognition that p53 function plays a key role in protecting humans from neoplasia and that half of all cancers lack p53 is to attempt to devise novel ways to exploit this knowledge in the development of new diagnostics and therapeutics for cancer. Several different approaches to this problem are now being evaluated in early clinical trials. These include the use of retroviruses and adenoviruses encoding wild type human p53 and the development of a defective adenovirus that can only replicate in cells that lack wild type p53 function. In biochemical experiments, agents have been discovered that can partially restore the DNA binding function and transcriptional activation function of certain point mutant p53 proteins. These agents act by affecting the allosteric regulation of the p53 core DNA binding domain, by the basically charged C terminal tail of the protein. In some human tumours p53 function appears not to be abrogated by mutation but instead is modulated by the expression of other proteins that bind to the p53 protein and neutralise its activity. Two of the best characterised cases in which this has been demonstrated are cervical carcinomas in which expression of the human papilloma virus E6 protein targets p53 to the ubiquitin degradation pathway so efficiently that the p53 response is neutralised. In the second case, seen in human sarcomas and gliomas p53 is neutralized by virtue of the high level expression of a p53 binding protein mdm2.

The Mdm2 protein

The mdm2 protein was first discovered as the predicted product of a gene that had undergone gene amplification and was present in a transformed mouse cell line on a double minute chromosome (hence mdm). The isolated gene proved capable of transforming fibroblasts. A year or so later the gene was rediscovered as encoding a putative p53 binding protein of 90,000 molecular weight that was present in anti-p53 immunoprecipitates of extracts from temperature sensitive mutant p53 transformed mouse cells. The availability of the cloned mdm2 gene led to a rapid set of discoveries that determined that the mdm2 protein bound the N terminus of the p53 protein and in model systems inactivated its function as a transcription factor and as a tumour suppressor protein. This led to the exciting concept that the mdm2 gene exerted its oncogenic affect by inactivating p53 in the same way as viral oncoproteins such as SV40 large T and adenovirus E1b protein had earlier been shown to act. The relationship between these two proteins became more involved when it was realised that the Mdm2 gene was transcriptionally activated by p53. This set up a feedback loop that could conceivably act to regulate negatively the
It was possible that the antibody failed to recognise of 10 amino acids near the N terminus of the protein. The epitope on p53 recognised by Bp53-19 was localised using a synthetic peptide library to a region of p53. The epitope on p53 recognised by Bp53-19 was complexed to Mdm2 although it was able to bind free Mdm2. It was found that a particular anti-p53 antibody called 3G5 would trigger the expression of the p53 reporter gene. We were at first very disappointed that our Mdm2 binding peptides did not induce such a response. It seemed possible that the antibody failed to recognise the complex by virtue of steric exclusion, that is Mdm2 masked the epitope on p53, or alternatively Mdm2 could have induced a conformational change in p53 that distorted the epitope. To distinguish between these possibilities it was necessary to determine whether Mdm2 bound to p53 at the Bp53-19 epitope. Despite an earlier publication having described that the Mdm2 binding region of p53 could not be reduced below about 30 amino acids it seemed worth trying Mdm2 binding assays against the synthetic peptide library. The library consisted of 15 amino acid peptides with four amino acids spacers linked to biotin. The library scanned the whole of the p53 sequence with a five amino acid stagger. The Mdm2 protein was produced in an insect cell expression system and a simple ELISA protocol used to determine whether the protein would bind to any of the p53 derived peptides. The results were immediately positive and very striking. A strong positive signal was obtained with only two peptides from the overlapping set suggesting that there was a single Mdm2 binding site on p53. This observation allowed rapid progress. Alanine scanning libraries, truncation libraries, and substitution libraries allowed the role of each amino acid in the Mdm2 binding motif to be defined.

This work was greatly enhanced by the use of phage peptide display libraries. With this method it was possible to screen millions of variant peptides for binding to Mdm2. We recovered a number of discrete phage from 15mer and 12mer peptide libraries that all showed homology to p53. This allowed us to define a minimum peptide motif needed for Mdm2 binding as FxxL/YWxxL. The four defined residues were all displayed on one face of an amphipathic alpha helix that fits into a hydrophobic groove on the surface of the Mdm2 protein. When we used peptide chemistry to analyse these phage-derived sequences in more detail we were delighted to find that one of the phage-derived peptide sequences (12/1) bound to p53 about 50 times more avidly than the natural p53-derived peptide.

Characterising the p53 Mdm2 interaction

Since Mdm2 acts to block the function of p53 it became very attractive to think about ways to disrupt this interaction in tumour cells that retained wild type p53 but expressed excess levels of Mdm2. In order to see whether this was feasible it was first necessary to study the nature of the p53 Mdm2 interaction. Using cells that expressed large amounts of the complex and a panel of monoclonal antibodies to p53 and Mdm2 it was found that a particular anti-p53 antibody called Bp53-19, failed to bind p53 when complexed to Mdm2 although it was able to bind free p53. The epitope on p53 recognised by Bp53-19 was localised using a synthetic peptide library to a region of 10 amino acids near the N terminus of the protein. It was possible that the antibody failed to recognise the complex by virtue of steric exclusion, that is Mdm2 masked the epitope on p53, or alternatively Mdm2 could have induced a conformational change in p53 that distorted the epitope. To distinguish between these possibilities it was necessary to determine whether Mdm2 bound to p53 at the Bp53-19 epitope. Despite an earlier publication having described that the Mdm2 binding region of p53 could not be reduced below about 30 amino acids it seemed worth trying Mdm2 binding assays against the synthetic peptide library. The library consisted of 15 amino acid peptides with four amino acids spacers linked to biotin. The library scanned the whole of the p53 sequence with a five amino acid stagger. The Mdm2 protein was produced in an insect cell expression system and a simple ELISA protocol used to determine whether the protein would bind to any of the p53 derived peptides. The results were immediately positive and very striking. A strong positive signal was obtained with only two peptides from the overlapping set suggesting that there was a single Mdm2 binding site on p53. This observation allowed rapid progress. Alanine scanning libraries, truncation libraries, and substitution libraries allowed the role of each amino acid in the Mdm2 binding motif to be defined.

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Proof of principle

The next key step in our study was to establish proof of principle that disruption of the p53 Mdm2 interaction with an Mdm2 binding peptide would actually trigger the activation of the p53 response. To do this we collaborated with Dr. David Wynford Thomas’s CRC group at the University of Cardiff. David’s laboratory had derived a human thyroid cell line that contained a p53 responsive reporter gene and expressed wild type p53 that was inactivated by the presence of Mdm2. David’s laboratory had shown (and we were able to confirm) that microinjection into the nuclei of these cells of an anti-Mdm2 antibody called 3G5 would trigger the expression of the p53 reporter gene. We were at first very disappointed that our Mdm2 binding peptides did not induce such a response. It seemed possible that the free peptides were too rapidly degraded so we sought a way to display and stabilise the conformation of the
Mdm2 binding peptides. The approach that we tried was to clone the peptides as in frame insertions into the active site loop of the well-characterized bacterial thioredoxin protein (Figure 2). We then analysed these thioredoxin insert proteins or TIPs for their p52 binding activity in ELISA assays. The TIPs mimicked p53 in binding tightly to Mdm2. Importantly the TIP that contained the phage derived sequence 12/1 bound even more avidly to Mdm2 than normal p53 could. Using this system we were readily able to construct control TIPs that contained mutant non-p53 interacting variants of the 12/1 sequence by substituting each of the key contact residues with alanine. Now we were at last able to test our hypothesis. We were delighted to find that microinjection of a plasmid encoding the TIP with the 12/1-insert peptide induced the p53 response whereas the control TIP encoding plasmid did not.

A control in normal cells reveals a new level of regulation

The p53 reporter response of the Mdm2 overproducing tumour cells to TIP-based disruption of the p53/Mdm2 interaction was very strong and so we decided to look in other cells to examine their response to this novel protein. When we injected normal fibroblasts containing a p53 reporter we were amazed to see a very strong reporter response to the TIP expression. Again the response was very specific and could be mimicked by injection of the 3G5 antibody. This was initially a confusing result. We knew that these cells contained only minute amounts of wild type p53 yet the level of the reporter response induced was very high. However we knew from previous studies that in response to DNA damage these cells produced high levels of p53 because of post-translational stabilisation of p53 and that these high levels of p53 induced a transcriptional reporter response similar to that induced by the TIP. It was therefore logical to look at the levels of p53 protein present in the TIP expressing cells. The results were very dramatic, the TIP that bound Mdm2 resulted in massive accumulation of p53 in the expressing cells. Microinjection of the anti Mdm2 antibody 3G5 also induced the same affect. The effect was very specific as another antibody to a different site on Mdm2 and could be mimicked by injection of the 3G5 antibody. This was initially a confusing result. We knew that these cells contained only minute amounts of wild type p53 yet the level of the reporter response induced was very high. However we knew from previous studies that in response to DNA damage these cells produced high levels of p53 because of post-translational stabilisation of p53 and that these high levels of p53 induced a transcriptional reporter response similar to that induced by the TIP. It was therefore logical to look at the levels of p53 protein present in the TIP expressing cells. The results were very dramatic, the TIP that bound Mdm2 resulted in massive accumulation of p53 in the expressing cells. Microinjection of the anti Mdm2 antibody 3G5 also induced the same affect. The effect was very specific as another antibody to a different site on Mdm2 and the control alanine mutant TIP induced no accumulation of p53 and no transcriptional reporter response. These results led us directly to propose a model whereby p53 is normally targeted for degradation by the Mdm2 protein. If this interaction is disrupted then p53 has a much longer half-life and accumulates in the cellular environment affected it. Specifically we had found that point mutant murine p53 proteins that were completely stable in human tumour cells that expressed endogenous wild type p53 were unstable in the environment of a cell containing endogenous wild type p53. However if these latter cells were treated with DNA damaging agents then both the endogenous wild type p53 and the introduced mutant p53 accumulated to high levels. When combined with the new insight from the TIP studies and the knowledge that the Mdm2 gene is transcribed in response to wild type p53 these earlier studies led to a new model for the stability of p53 in tumour cells. The model proposed that tumour cells that lack functional p53 lack Mdm2. It is this lack of Mdm2 that in tumour cells makes the p53 a stable protein. The great attraction of this model is that it links a lack of protein transcriptional function to p53 stability and therefore explains why mutant p53 proteins are stable as are wild type p53 proteins that have been inactivated for example by complexing to SV40 T antigen. The straightforward test of this model was to reintroduce Mdm2 into cells that contained stable mutant p53. Using both microinjection of Mdm2 expression constructs and the establishment of stable Tet inducible Mdm2 expressing cell lines we confirmed that point mutant p53 proteins are still substrates for Mdm2 dependent degradation. More recently other requirements for p53 to act as a substrate for Mdm2 dependent degradation have emerged. The proteins need to be tetrameric and also require an intact C terminus. As yet the reasons for these additional requirements have not been determined.

Activation of the p53 response by different modulations of the Mdm2 pathway

The key hypothesis of the current model is that p53 is stabilised in response to stress signals by alteration of the rate of mdm2 dependant degradation. Very recent studies have supported this model but also shown that different activators of the p53 response target the Mdm2 degradation pathway by completely different mechanisms. Ionising radiation for example seems to act through the ATM and ATR kinases to phosphorylate p53 and Mdm2 and reduce complex assembly. UV radiation by contrast seems to reduce the levels of mdm2 mRNA present in cells. In a most exciting development activated oncogenes such as Ras and adenovirus E1A seem to trigger the p53 pathway by stimulating the production of a small protein p19 

Substrate requirements for Mdm2 dependent degradation

Our earlier studies had shown us that the stability of mutant p53 proteins was not intrinsic, rather the
tion and regulation. The rewards of that effort are now becoming apparent in the discovery of many new approaches to cancer therapy. These include current gene therapy trials using wild type p53 producing viruses and the clever Onyx development that uses a disabled virus that can only replicate in p53 mutant cells. The realisation that many classic cytotoxic anti-cancer drugs and radiation treatments work at least in part through p53 dependent pathways has suggested that they might be replaced by non-toxic activators of the p53 response. There is, for example, a large effort underway in many pharmaceutical companies to identify low molecular weight compounds that might mimic our TIP and activate the p53 response by blocking p53-Mdm2 interaction. Attempts are also being made to use anti-sense inactivation of Mdm2. These approaches might work in tumours that retain wild type p53 but in which p53 has been inactivated by another mechanism. Here the tumours that lack Arf look very promising since the TIPs are able to activate the p53 response in such cells.

In those cases in which p53 is lost by mutation however such concepts would not work. However it is conceivable that even here progress could be made. For example it may be possible to produce a toxic drug that is inactivated by Mdm2. Such a drug or agent would be safe in normal cells that contain wild type p53 and therefore express active Mdm2 but would accumulate to toxic levels in cells that had mutant p53.

There is of course enormous amount more work to be done in defining how p53 is regulated and what downstream functions it exerts on the cell. We are still unclear for example about how important transcriptional repression as opposed to activation is for p53 function. Some also claim functions of p53 distinct from transcriptional regulation and while these data are not compelling some direct involvement of p53 in for example DNA repair signalling complexes cannot yet be excluded. It is hard not to be optimistic that by the Campaigns 100th anniversary we will finally see the promise of molecular oncology to improve the treatment of Cancer realised in the clinic.

References

2. Lane DP. Cancer, p53, guardian of the genome [news; comment] [see comments]. Nature 1992; 358:15-6.
Embryonic beginnings of definitive haematopoietic cells

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Introduction

The adult haematopoietic system is a complex series of pluripotent, multipotent and unipotent cellular intermediates which proliferate and differentiate into at least eight morphologically and functionally distinct mature blood cell types. Haematopoietic stem cells are at the foundation of this cellular hierarchy in the adult. The cells within this hierarchy have been defined by numerous in vitro and in vivo haematological assays which measure proliferation and lineage differentiation. The most stringent of the assays, the radiation chimera assay, has been used to demonstrate the presence of pluripotent haematopoietic stem cells in bone marrow and foetal liver. In vivo transplantation of true adult-type haematopoietic stem cells into adult recipient mice depleted of endogenous haematopoietic stem cells by a lethal dose of irradiation leads to the complete, long-term engraftment of all blood lineages by donor-derived stem cells. Thus, pluripotent haematopoietic stem cells possess the following complex characteristics: 1) potential for all haematopoietic lineages as demonstrated by clonal markers; 2) high proliferative potential leading to 100% donor-derived engraftment; 3) long-term activity throughout the lifespan of the individual; and 4) self-renewal as demonstrated by in vivo serial transplantations. The clinical importance of stem cells with these characteristics is widely recognised in transplantation scenarios for blood-related genetic deficiencies and leukaemias.

The developmental origins of adult-type haematopoietic stem cells in mammals are of current interest. In developmentally early haematopoietic microenvironments, stem cells are induced and/or subsequently expanded from cells derived from the mesoderm germ layer. Subsequently, haematopoietic stem cells colonise the liver during foetal stages and the bone marrow of the adult where they remain throughout life. Early haematopoietic development has been examined in both mammalian and non-mammalian vertebrate embryos. Mammalian vertebrates have the advantage of many advantages in vitro and in vivo assays for defining the presence of an array of haematopoietic progenitors and stem cells within the embryo. Primitive erythroid cells, erythroid-myeloid progenitors, CFU-S and haematopoietic stem cells have been found in the yolk sac at E7, E8, E8.5 and E11 respectively. These results led to the previously accepted notion that the yolk sac was the source of the adult haematopoietic system in mammals. However, orthotopic grafting experiments which can be performed with the large embryos of avian or amphibian species showed that while the yolk sac or ventral blood islands (yolk sac analogue) produced haematopoietic cells, these were transient blood cells present predominantly during embryonic stages. The long-lived adult haematopoietic system was generated instead by the cells in an intraembryonic site surrounding the dorsal aorta and pro/mesonephros. The dichotomy in these results of higher and lower vertebrates persisted for over twenty years leading to the assumption that mammalian haematopoietic development is different from that in non-mammalian vertebrate species. With the absence of previous studies examining this intraembryonic site in mammals, we set out to determine whether haematopoietic activity was present and could have its origins in the region comprising the dorsal aorta, gonads and mesonephros (AGM) of the early and mid-gestation mouse embryo.

The AGM region contains haematopoietic progenitors and stem cells

To examine the haematopoietic potential of the intraembryonic AGM region we first performed the short-term in vivo spleen colony-forming unit (CFU-S) assay. We injected a single cell suspension of various tissues from E8, E9, E10, or E11 mouse embryos into lethally irradiated adult female recipient mice and examined their spleens at 9 and 11 days post-transplantation for the presence of macroscopic (erythroid-myeloid) colonies. The donor injected cells were obtained from male embryos or transgenic embryos marked by either a Y chromosome or a human beta-globin gene locus respectively. While E8 yolk sac, AGM, liver and blood contained no CFU-S, such progenitors are present in both E9 yolk sac and AGM. At E10 the number and frequency of CFU-S in the AGM region surpasses that in the yolk sac and peaks at late E10. Thereafter, CFU-S decrease rapidly in the AGM, with a concomitant increase in CFU-S in the liver. These results demonstrate the presence of potent multilineage progenitors in the AGM region, and their temporal and spatial distribution suggest that CFU-S from the AGM region colonise the liver.

In addition to these data on the multipotent CFU-S progenitor, Godin and colleagues found that the intraembryonic para-aortic splanchnodeulea (PAS) at E8.5 contains progenitors for the B1a subset of B-lymphocytes by transplantation of this embryonic region under the kidney capsule of severe combined
With these convincing data that multipotent haematopoietic progenitors could be found in the PAS/AGM region, long-term mouse radiation chimeras were generated to test whether the AGM region contains pluripotent adult-type haematopoietic stem cells. Transplantations were performed as described for the CFU-S experiments, and mice were tested for engraftment at greater than 4 months post transplantation. No donor-derived engraftment was observed for E8 or E9 yolk sac or AGM. However, transplanted E10 AGM region cells were able to fully engraft 3 out of 100 recipients, while E10 yolk sac resulted in no engraftment. Even at eight months post-transplantation, E10 AGM region cells can re-populate the blood system of the recipients up to 100% in all haematopoietic tissues and lineages. Secondary and tertiary serial transplantations of bone marrow from these recipients indicated that the E10 AGM haematopoietic stem cells are self-renewing. Moreover, at E11 the AGM region contains a high frequency of such haematopoietic stem cells as demonstrated by the complete long-term engraftment of 11 out of 19 recipients. E11 yolk sac and liver also contained haemopoietic stem cells but at a lower frequency. Recently Yoder and colleagues have shown that at E9 both the yolk sac and AGM region contain multipotent progenitors that can in vivo re-populate neonatal but not adult recipient mice. These data suggest that the earlier progenitors in the yolk sac and AGM have some but not all the characteristics of adult haematopoietic stem cells. Thus, the AGM region appears to be the first site within the mouse conceptus to initiate fully competent adult-type haematopoietic stem cells.

The AGM region autonomously initiates haematopoietic stem cell activity

At E10 the AGM region contains the first haematopoietic stem cells at limiting numbers. However, it is possible that these stem cells are generated at another site within the mouse conceptus and quickly migrate through the circulation or interstitially to localise in the AGM region. The circulation between the yolk sac and the embryo body is established at E8.5 in gestation. Thus, to examine the site of initiation of the first haematopoietic stem cells, we instituted an organ explant culture step for AGM, yolk sac or liver before the in vitro transplantation assay. Two to three days of organ culture of individual AGM, yolk sac or liver explants alleviates any cellular exchange between these tissues. When we examined E9 cultured tissues, no haematopoietic stem cells were found in AGM, yolk sac or liver. The first haematopoietic stem cells appeared in E10 cultured AGM explants. Quantitatively these stem cells outnumbered those found in uncultured AGM by a factor of 15, strongly suggesting the induction and/or expansion of haematopoietic stem cells during the 3-day in vitro culture period. Interestingly, no haematopoietic stem cells were found in E10 yolk sac or liver but began to appear in E11 and late E11 cultured yolk sac and liver, respectively. These results show the autonomous and exclusive production of haematopoietic stem cells at E10 by the AGM region and suggest the colonisation of liver by AGM-derived haematopoietic stem cells. The yolk sac may also be colonised by AGM-derived haematopoietic stem cells and/or endogenously generate its own haematopoietic stem cells beginning at E11.

A similar organ culture step was used to determine the site of generation of the first multipotent (lymphoid-myeloid-erythroid) progenitors. At E7.5 and before the circulation is established between the embryo body and the yolk sac, these progenitors are found only in the para-aortic splanchnopleura. The yolk sac contains such progenitors only after E8.5. Thus, these definitive haematopoietic progenitors as well as the first fully competent adult-type haematopoietic stem cells are autonomously generated within the embryo body before they appear in the yolk sac.

The first haematopoietic stem cells are C-Kit+CD34+ and localise to the anterior AGM

Phenotypic characterisation of the AGM haematopoietic stem cells was performed. Cell surface markers indicative of adult-type haematopoietic stem cells were found on AGM haematopoietic stem cells. When transplanted at various doses, E11 haematopoietic stem cells were always found in the c-kit+CD34+ double positive population. The E9 yolk sac and AGM cells that possess the ability to repopulate conditioned new born mice (but not adult recipients) to low levels are also c-kit+ and CD34+. However, the Sca-1 haematopoietic stem cell marker, which is a marker of the haematopoietic stem cells of foetal liver and adult bone marrow but not yolk sac haematopoietic cells, is expressed on E11 AGM haematopoietic stem cells suggesting this is a distinctive marker of only true definitive haematopoietic stem cells. Interestingly c-kit-CD34+ E11 AGM haematopoietic stem cells are negative for all mature lineage markers except Mac1 at 121. While fifty percent of the AGM stem cells were found to be Mac1-negative, the other fifty percent were Mac1-positive and have been suggested to be the subset of AGM stem cells ready to colonise the foetal liver (at E11, E12 and E13) all foetal liver haematopoietic stem cells are Mac1-positive. These marker studies strongly suggest a direct lineage relationship between the haematopoietic stem cells of the AGM region and the foetal liver and, together with the results of the organ culture studies, suggest a colonisation of the foetal liver with AGM-generated haematopoietic stem cells. Using a transgenic mouse expressing the LacZ reporter gene in Sca-1 expressing cells, we examined the localisation of Sca-1-LacZ-positive cells within intact AGM tissue. When E10, E11 and E12 embryos were examined, β-galactosidase-positive cells were found in the anterior portion of the AGM region. The cells lining the pronephric and mesonephric tubules stained brightly, while the cells in surrounding mesenchyme stained to an intermediate level. All yolk sac

immunodeficiency (SCID) mice. In a two-step culture system multipotent progenitors for the B- and T-lymphoid lineages as well as erythroid-myeloid lineages were found in the PAS at E8.5. These cells were found to be positive for the AA4.1 surface marker previously found on foetal liver haematopoietic progenitors and stem cells.

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cells were negative. Moreover, the kinetics of the AGM β-galactosidase staining coincided with the presence of haematopoietic stem cells in the AGM region, thus suggesting a localisation for the first stem cells. By using the organ culture system we also tested whether we could further localise the first functional haematopoietic stem cells to the anterior and/or posterior portion of the AGM. In two experiments we found all haematopoietic stem cell activity in the anterior portion of cultured E10 AGMs. Hence the β-galactosidase-positive cells may provide a precise localisation for the first haematopoietic stem cells in the AGM region, although at this time it is undetermined whether the β-galactosidase-positive cells in the mesonephric tubules or the cells within the surrounding mesenchyme (which stain intensely) are the functional stem cells. Further functional experiments are in progress to examine which positive population contains the haematopoietic stem cells.

Conclusions and future directions

The results of our in vivo transplantation experiments using the mouse as a model for mammalian haematopoiesis clearly demonstrate the potency of the AGM region in the initiation of adult-type haematopoietic stem cells. The AGM region autonomously and exclusively initiates the first adult-type haematopoietic stem cells at E10, one day earlier than these cells can be found in the yolk sac. Previously, in contrast to the intraembryonic source found in non-mammalian vertebrates, the yolk sac was thought to be the generating source of these stem cells in mammals. The discovery of the haematopoietic potential of the AGM region now yields a clear case for strong similarities in developmental haematopoiesis between all vertebrate species. It appears that the mouse yolk sac participates predominantly in the generation of the embryonic haematopoietic system, while the AGM region is dedicated to the initiation and production of the adult haematopoietic system.

Indeed this appears to be reflected in the genetic programming of cells destined to become embryonic or adult haematopoietic cells. Recent advances in targeted mutagenesis in mice have yielded results that demonstrate the differential requirement for some genes (AML-1, GATA-2, GATA-3, c-kit) in adult foetal liver haematopoiesis but not embryonic haematopoiesis. In contrast the mutation of other genes (flk-1, tal-1/SCL) results in the impairment of haematopoiesis in both the yolk sac and foetal liver. Thus, the genetic programmes of embryonic and adult haematopoietic cells appear to overlap initially during stages determining haematopoietic fate, but become unique as more complex programming is required in cells destined to become part of the adult haematopoietic system. It may be predicted that the primary cellular defects of the genes, affecting foetal liver haematopoiesis occur in the induction, expansion or maintenance of haematopoietic stem cells and progenitors in the AGM region before they colonise the foetal liver. We are currently examining the roles of such genes in the AGM region and in the generation of the adult haematopoietic system by organ explant cultures and radiation chimera approaches.

Finally, the question remains how are the first fully competent adult-type haematopoietic stem cells generated in the AGM region? As the foetal thymic organ culture system has yielded great advances in knowledge concerning thymocyte differentiation and development, we hope to make use of chimeric AGM organ cultures to determine whether the precursors for the first haematopoietic stem cells are generated in situ within the AGM region or are recent emigrants from other embryonic sites such as the yolk sac. These cultures will also be used to test whether adult bone marrow haematopoietic stem cells can be increased in number in the AGM microenvironment. Recent findings of Mukouyama et al. suggest that haematopoietic progenitors from the AGM region can, indeed, be increased in vitro. Hence, our future investigations will include differential cloning to address what are the unique signals within the AGM microenvironment that lead to the induction and/or proliferation of the first fully competent adult-type haematopoietic stem cells.

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References

10. Turpen JB, Knudson CM, Hoefen PS. The early ontogeny of hematopoietic cells studied by grafting cytotogenetically labelled tissue anlagen: localization of a


Stem cell leukaemia gene and its role in normal and leukaemic haematopoiesis
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SCL is frequently dysregulated in human T-ALL. The stem cell leukaemia (SCL) gene was first identified because of its involvement in a chromosomal translocation in an unusual human stem cell leukaemia. In this leukaemia, the lymphoid leukaemic cells underwent a dramatic transformation, and acquired features of myeloid cells in response to treatment with 2'-deoxycoformycin. Before and after this phenotypic conversion the leukaemic cells carried a translocation between chromosome 1p32-33 and the T-cell receptor (TCR) \( \alpha/\delta \) locus on chromosome 14q11. Using probes to the human TCR \( \delta \) locus, the translocation breakpoint was isolated and a new gene on chromosome 1 was identified.1,2 The name Stem Cell Leukaemia gene or SCL was assigned, a remarkably appropriate name given the essential function of this gene in normal haematopoietic stem cells. The chromosomal translocation was the result of aberrant activity of the DNA-recombinase enzyme complex which had inadvertently joined chromosomes 1 and 14 together. The same gene was independently identified by several other groups, again because of its involvement in human T-cell acute lymphoblastic leukaemia (T-ALL).

There are at least two factors that partly explain the error on the part of the DNA-recombinase enzymes that generated the translocation. Firstly, the recombinases normally recognise specific DNA sequences that flank the segments of DNA to be rearranged. These serve as specific signals that allow the enzymes to identify correctly and juxtapose segments of DNA in the process of assembling a functional TCR (or immunoglobulin) gene. Very similar sequences are present in several regions of the human SCL gene but intriguingly are not seen in equivalent regions of the mouse gene.1 It is not known why this is so. However, the presence of signal-sequences in the human SCL gene does provide a site for rearrangements to occur. Secondly, it is now clear that SCL is crucially important at the earliest stages of haematopoietic stem cell development and differentiation. The SCL gene was likely therefore to be hypomethylated in an open, chromatin configuration thus making it vulnerable to the recombinase enzymes.

While SCL expression is not normally detected in the vast majority of normal thymocytes it is expressed in leukaemic T-lymphocytes. The majority of translocations involving SCL primarily occur into the TCR \( \alpha/\delta \) locus, however translocations involving SCL and chromosomes 3, 5, and 7 (the TCR \( \beta \) locus) have also been documented. These translocations all have in common the likely involvement of the recombinase enzyme complex and the aberrant expression of SCL as a result.3 Because of the frequent involvement of SCL in leukaemias with a T-cell phenotype the names TCL-5 and TAL-1 were also proposed.4

There is a second mechanism by which the SCL gene can be disrupted and aberrantly activated in leukaemic T-lymphocytes. This involves deletions of approximately 90kb of DNA from chromosome 1 that brings SCL under the control of the upstream, ubiquitously expressed SIL (for SCL Interrupting Locus) gene.4 Again recombinase enzymes are probably responsible for the SIL/SCL deletions (also known as TAL5): the presence of specific recombination signal sequences in both the SIL and SCL genes ensures that a limited number of SIL/SCL deletional events are seen recurrently in independent leukaemic samples. The SIL/SCL deletional event in T-cell leukaemia occurs in up to 30% of cases.1 Each leukaemia has unique sequences at the site of the SIL/SCL junction which can serve as a useful leukaemia and patient-specific marker. Although it was proposed that SCL was expressed in up to 60% of childhood T-ALL samples without rearrangements of the SCL gene, this is probably incorrect and due to contaminating normal cells.

There is no distinct clinical subgroup of T-ALL associated with SCL rearrangement, however deletion of one or both TCR \( \delta \) gene(s) is frequently observed. Thus SCL rearrangements are seen predominantly in CD3 positive, TCR \( \alpha/\beta \) positive T-ALL.

Structural alterations of the SCL gene are not seen in B-lineage ALL, T-cell non-Hodgkin’s lymphomas nor in a range of other solid tumours. Although expression of SCL has been observed in samples from patients with acute myeloid leukaemia, this probably reflects malignant transformation of myeloid progenitor/stem cells which normally express SCL.5,6

SCL and its Role in the Genesis of Leukaemia
The mechanism(s) by which SCL contributes to leukaemia development remains uncertain. There is evidence that SCL can provide a proliferative advantage to cells. For example, interfering with SCL function in a multipotent human cell line decreased proliferation, progression through the cell-cycle and self-renewal potential.7 Similarly aberrant SCL expression in T-cells increased clonogenic ability of the cells. In murine myeloid cells, retrovirally-enforced SCL ex-
pression allowed cells to overcome growth-factor-induced suppression of proliferation. Likewise, SCL expression caused by insertion of a retroviral-like element conferred a growth advantage on myeloid cells and resistance to differentiation-inducing agents. Thus in a variety of cellular contexts enforced SCL expression stimulates proliferation and opposes differentiation. In a different cellular context, erythroid cells, enforced SCL expression also enhances cellular proliferation but here differentiation is also stimulated.1,9

SCL expression also delays apoptosis and again this action may depend on the cell type in which SCL is acting. Therefore it is likely that SCL contributes to leukemogenesis by multiple mechanisms that include an increase in proliferation, self-renewal potential and a decrease in cell death.

These actions of SCL translate into increased leukaemia in a variety of animal models. In some transgenic models expression of SCL did not cause leukaemia, presumably as a consequence of the regulatory elements that were used. However, several groups have demonstrated co-operation in vivo between SCL and a variety of additional genes including ABL, casein kinase II, p53 and RAS.1 SCL also collaborates with the LIM domain proteins LMO-1 and ABL, casein kinase II, p53 and RAS.1 SCL also collaborates with the LIM domain proteins LMO-1 and LMO-2 to generate tumours.15 This interaction is particularly significant because SCL, LMO-1 and LMO-2 are all implicated in human T-ALL. However, all the mouse models suggest that additional events are still needed, and in the context of SCL and the LIM domain proteins, defects in the DNA mismatch repair gene MSH2 are probably important.

The biochemical basis for the tumorigenic co-operation is unclear. However, it is known that SCL interacts with the products of the E2A gene11,12 and related proteins to form heterodimeric DNA-binding complexes. The heterodimer can form part of a larger protein complex in which LMO-2 acts as a molecular bridge between the zinc finger transcription factor GATA-1 and the SCL/E2A heterodimer.14 Using a chromatin immunoprecipitation approach Romeo and colleagues14 recently identified a gene of unknown function, expressed in erythroid cells and directly regulated by a complex containing SCL and GATA-1. Although these interactions have been characterised primarily in erythroid cells, where SCL is normally expressed, a large protein complex occurs in T-cells but with altered DNA binding specificity. The constituents of the complex are likely to vary in different cell types, differentiation states or cell cycle stages, perhaps explaining in part the multiplicity of functions ascribed to SCL and other transcription factors.

The leukemogenic effect of SCL expression in T-cells may reflect either activation of target genes that should normally be silent, or alternatively the sequestration of other components of the multi-protein complex. Several lines of circumstantial evidence support the latter view:

1. SCL can sequester E2A proteins thereby inhibiting transactivation of genes, and mice lacking the E2A gene are known to develop T-cell tumours.
2. Casein kinase II co-operates with SCL to hasten T-cell tumours, and casein kinase II is also known to inhibit activity of E2A proteins.

3. Mice carrying an SCL transgene lacking its transactivation domain still develop lymphoid tumours.

If SCL acts as a molecular ‘sink’ in T-ALL, this predicts that other transcription factors capable of interacting with E2A proteins could be leukemogenic in T-cells. This is true of the proteins LYL-1 and TAL-2 that are rarely involved in translocations in T-ALL.1 The prevalence of SCL rearrangements in T-ALL presumably reflects the presence of the specific signal sequences for DNA-recombinases in the human SCL gene.

SCL Normally Functions as a Critical Regulator of Haematopoiesis

Given the stem cell phenotype of the initial leukaemia, it seemed possible that the SCL protein could be important in normal haematopoietic cells. The first evidence for this came from analysis of the amino acid sequence. Two important domains were evident. One is an helix-loop-helix (HLH) region common to a large family of functionally important transcription factors. This region allows SCL to heterodimerise with products of the E2A gene, themselves HLH transcription factors. The second domain is a basic region, present within SCL and other HLH proteins that is required for DNA binding. The site on the DNA to which the SCL/E2A heterodimer binds is an E-box motif, which is also common to other HLH proteins. Surprisingly, recent studies have demonstrated that SCL can exert at least some of its functions without this domain.15

A role for SCL in the regulation of normal haematopoiesis was suggested by its normal expression pattern; SCL is restricted to haematopoietic cells, endothelial cells, the central nervous system and embryonic skeleton.1,16 Within the haematopoietic compartment SCL is normally expressed in erythroid cells, mast cells, megakaryocytes and progenitor/stem cells.1

The first clue to the normal function of the SCL protein came from antisense experiments demonstrating that SCL regulates proliferation and self-renewal in a multipotent haematopoietic cell line.6 A positive role for SCL in erythroid differentiation was suggested by the increase in SCL mRNA levels during erythroid differentiation6 although a concurrent fall in levels of SCL protein implies additional levels of control. A more direct role for SCL in erythroid differentiation was provided by gene delivery experiments in which enforced expression of SCL enhanced erythroid differentiation of haematopoietic cell lines. Conversely as cells differentiated along the myeloid/macrophage pathway, levels of SCL mRNA and protein decreased, becoming essentially undetectable. This fall in SCL expression is necessary since enforced SCL expression prevents macrophage differentiation from proceeding normally. These results in cell lines have recently been confirmed and extended using normal human CD34-positive stem cells. In these cells, retroviral-directed SCL expression enforces and hastens erythroid and megakaryocytic differentiation, with less striking effects on the myeloid compartment.6,8

Dramatic confirmation that the SCL gene encodes...
a vital stem cell regulator came from studies in which SCL function was destroyed. This was achieved using gene targeting in embryonic stem (ES) cells to derive SCL-null mutant animals (knock-out mice). Mice lacking SCL function died at about 8.5 days of embryonic development (E8.5) with a complete failure of yolk sac haematopoiesis, suggesting that SCL is crucial for the generation of haematopoietic cells in the early embryo (primitive haematopoiesis). SCL plays a similar essential role in adult, definitive haematopoiesis. Following injection of SCL-null ES cells into blastocysts, analysis of the resulting adult chimeric animals showed the complete failure of the SCL-null ES cells to contribute to haematopoietic cells, although the mutant ES cells did contribute to all other tissues examined.17,18 The SCL-null ES cells also failed to give rise to haematopoietic cells during in vitro culture and failed to express haematopoietic specific genes. Taken together, these observations demonstrate that SCL plays a critical role in the generation and/or subsequent behaviour of pluripotent haematopoietic stem cells. This function of SCL is cel-autonomous, and occurs within the stem cell itself. These results also predict that normal SCL-expressing progenitors should function as multipotent cells, displaying the ability to generate haematopoietic cells committed to multiple lineages. This prediction has recently been confirmed using a gene targeting approach; a marker gene (LacZ) was introduced into the SCL locus thereby allowing the isolation of SCL-expressing cells for functional analysis. These results confirm the status of SCL as a ‘master regulator’ for haematopoiesis.15

The results with SCL are remarkably similar to those seen when the LM O-2 gene is destroyed. LM O-2 null animals also lack any detectable haematopoietic cells within the yolk sac and embryo, and die at about E8.5. Similarly, LM O-2 null ES cells fail to contribute to adult haematopoiesis thus reinforcing the parallels between SCL and LM O-2. In addition to interacting in erythroid and some leukaemic T-cells, it therefore seems likely that SCL and LM O-2 are components of a critical multi-protein complex present in haematopoietic stem cells. In light of these results, one interesting gene that appears to be directly regulated by SCL is c-kit, the gene encoding the receptor for stem cell factor (SCF).

Given the crucial function of SCL within haematopoietic stem cells, the expression of SCL within endothelial cells raised the possibility that SCL could also function within an even more primitive cell, the putative common haematopoietic and endothelial precursor, the haemangioblast. There is a long-recognised, close relationship between the development of blood and endothelium, suggesting that there may be such a common precursor.

Direct evidence for a role for SCL in haemangioblast formation came when SCL was co-expressed with Flk-1 in presumptive haemangioblasts within early postero-lateral mesoderm of zebrafish embryos.19 Ectopic expression of SCL mRNA during zebrafish development resulted in an expansion of haematopoietic and endothelial precursors at the expense of somitic and pronephric duct tissues. Taken together, these data demonstrate that SCL is capable of specifying haemangioblast development from early mesoderm.

It is interesting to contrast the loss of function phenotype, in which endothelial cells develop but fail to contribute normally to vessel formation, with the gain of function experiments described above. These results are consistent with previous studies of HLH proteins in myogenesis and neurogenesis suggesting a cascade of HLH proteins that can display overlapping functions and expression patterns. In the case of SCL, functional redundancy with related HLH proteins may explain the relatively normal development of endothelial cells in SCL null mice.

SCL, how is it regulated?

The mechanisms by which SCL is controlled are relatively poorly understood. However the importance of these mechanisms is amply demonstrated when they go awry in the T-lymphocyte.

At the level of SCL protein, there is a requirement for HLH partner proteins such as E12 and E47. Additional control is provided via a sub-group of HLH proteins that lack a DNA-binding basic domain and thereby isolate other HLH proteins in inactive complexes. Moreover, the SCL protein itself exists in several different forms as a result of multiple sites of protein initiation within the SCL gene.

There is evidence linking SCL to several signal transduction pathways. These include BMP-4 and its receptor, SCF, erythropoietin and growth factors that induce macrophage differentiation.20 Analysis of the cis-regulatory sequences in the SCL gene has defined two promoter elements, one of which is regulated by GATA-1, SP1 and SP3 and is active in transient reporter assays in erythroid and mast cells. The other, promoter 1b, is regulated by PU-1, SP1 and SP3 and is active in mast and primitive myeloid cells. In addition there are distant regulatory elements (enhancers) in the SCL gene that are able to direct SCL expression within the brain and within primitive haematopoietic cells.20

Conclusions

SCL is clearly pivotal for normal haematopoiesis and vasculogenesis and is also likely to have an important role in the nervous system. It is also a crucial factor in the development of human T-cell leukaemia. However many questions remain unanswered. How does SCL induce an undifferentiated mesodermal cell to become a haemangioblast and thence a multipotent haematopoietic stem cell? Does SCL perform the same role in different classes of haematopoietic stem cells throughout ontogeny? What is the role of SCL in the nervous system? Why is ectopic SCL expression leukemogenic in T-cell progenitors? To address these questions, advances in at least three areas are required. Firstly, we need to understand the significance of the multiprotein complexes that include SCL. Do they differ in distinct cell types and to what effect? Secondly, it will be essential to identify direct target genes downstream of SCL in different SCL-expressing cell types. Thirdly, how is SCL itself regulated? What triggers SCL expression in early mesoderm and during differentiation along specific haematopoietic lineages? Answers to these questions will give further insights into the function of this important regulator.
Acknowledgments
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References

13. Wadman IA, Osada H, Grutz GG, et al. The LIM-only protein Lmo2 is a bridging molecule assembling an erythroid, DNA-binding complex which includes the TAL1, E47, GATA-1 and Ldb1/NL1 proteins. EMBO J 1997; 16:3145-57.
Transcriptional regulation of myelopoiesis and myeloid leukaemia

Transcription factors play a major role in haematopoietic development in general and in differentiation in particular. This is especially important in the myeloid lineage, in that the most common form of acute leukaemia in adult humans results from a block in normal myeloid differentiation, and many of the abnormalities critical to development of myeloid leukaemias involve transcription factors. In this manuscript, we will not attempt to be exhaustive, but rather to update the reader on newer concepts of myeloid lineage commitment and differentiation which have emerged in the past couple of years with a focus on two myeloid transcription factors, PU.1 and C/EBPα. The reader is referred to previous reviews for a more thorough background on previously published data, particularly reference. Recent studies have emphasised several themes in terms of myeloid differentiation. These include mechanisms of downregulation of alternative pathways, as well as the increasingly important role of transcription factor interactions in lineage commitment decisions. We will try to highlight examples of these two mechanisms in this chapter.

How does a stem cell decide to commit to the myeloid lineage?

Previous models have described stochastic, deterministic, and combination models of haematopoietic lineage commitment. Our view of myeloid commitment combines the two, but in a manner different from traditional older views. Several recent studies have been added to previous studies suggesting that growth factor signalling does not determine myeloid development. There is also accumulating data supporting the idea that differentiation is determined by deterministic models in which the determination factor is a transcription factor, not an external growth factor. (Figure 1; Table 1). Recent models have proposed that stem cells or early multipotential progenitor cells may co-express low levels of lineage specific transcription factors, such as GATA-1 and PU.1. Some event (and this may represent the stochastic aspect of differentiation) leads to an increase in expression and/or activity of one or more of these transcription factors. Examples of possible initiating (or destabilising) events include interactions with stroma, or perhaps local differences in concentrations of a growth factor leading to activation of specific growth factor pathways. An important concept is not only the amount of any given transcription factor, but whether such an initiating event leads to a relative increase in expression of one of these factors over the other. For example, treatment of CD34+ cells with GM-CSF leads to increases in PU.1 and decreases in GATA-1 mRNA. PU.1 autoregulates its own promoter and activates the gene for the GM-CSF receptor α, leading to viability and proliferation of early myeloid progenitors (Figure 1). In contrast, increases in GATA-1 induced in the same cells by erythropoietin could potentially lead to autoregulation, activation of the erythropoietin receptor, and downregulation of PU.1 expression. Importantly, abnormal expression of these factors in the wrong lineage can result in a block of differentiation. For example, overexpression of GATA-1 in early myeloid cells blocks myeloid development, and overexpression of PU.1 can block erythroid differentiation and lead to erythroleukaemia. Therefore, inhibition of pathways is also an important concept in understanding myeloid development (Table 2).

The role of protein-protein interactions in myeloid lineage commitment

An important emerging concept is that not only is the relative expression of transcription factors important, but that interactions among and combinations of factors may be critical. A number of such interactions have been described previously. Most of these have resulted in positive activation of myeloid promoter activity. Very recent results have indicated that factors such as GATA-1 and GATA-2 interact with PU.1 to inhibit its function and vice versa. Both GATA-1 and GATA-2 can interact with PU.1 through a relatively small region within the PU.1 Ets domain. Interestingly, this same region of PU.1 can interact with AM L1, c-Jun, and C/EBP factors, and therefore comprises an important interaction domain of PU.1 mediating both positive and negative regulation of PU.1 activity (Figure 2). GATA does not inhibit PU.1 DNA binding activity or the amino terminal transactivation domain, but rather GATA competes for c-Jun, a co-activator of PU.1 function, for binding to the PU.1 interaction domain (Figures 2, 3). PU.1 can also inhibit GATA function in early erythroid cells, and this effect is mediated by inhibition of GATA DNA binding through an interaction with PU.1. This interaction with GATA is not through the same small region in the PU.1 Ets domain, but rather through binding of the PU.1 amino terminus to the GATA carboxyl ter-

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minal zinc finger. Such complementary inhibitory functions could play an important role in early decisions of stem cells to become myeloid cells (Figure 3). In non-myeloid cells, such as stem cells and/or erythroid cells, GATA-2 and GATA-1, if present in excess, could inhibit PU.1 function by competing for binding of c-Jun, a co-activator of PU.1 function. In myeloid cells, PU.1 is more highly expressed than the GATA proteins, leading to inhibition of GATA function.

Lineage specific coactivators and myeloid development
It is likely that in the next few years a number of myeloid specific co-activators will be discovered. A very important example of this type of protein is the D. Tenen

Table 1. Hypotheses regarding myeloid development.

<table>
<thead>
<tr>
<th>Hypothesis</th>
<th>Traditional</th>
<th>“Transcriptocentric”</th>
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<tr>
<td>Stochastic</td>
<td>Chance</td>
<td>Chance encounter of early progenitor with stroma or CSF, leading to activation</td>
</tr>
<tr>
<td>Deterministic</td>
<td>CSFs induce differentiation</td>
<td>Transcription factors determine differentiation, modulated by expression and interactions with other factors</td>
</tr>
<tr>
<td>Stochastic/Deterministic</td>
<td>Combines both</td>
<td>Stroma and/or CSF signals induce transcription factor expression and/or activation</td>
</tr>
<tr>
<td>Default</td>
<td>Erythroid/monocytic</td>
<td>Transcription factors modulate default pathways (e.g. C/EBPα directs myeloid precursor away from default monocytic pathway)</td>
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GATA co-activator FOG, which is specific for erythroid cells and critical for GATA function. FOG itself does not bind to DNA, but interacts with the amino terminal zinc finger of GATA-1 and is essential for GATA function in induction of erythropoiesis. 18

A recent example of a potentially important myeloid co-activator is c-Jun. 17 While c-Jun is not as specifically expressed as FOG, there is evidence to suggest that it does demonstrate very specific patterns of expression. The c-Jun proto-oncogene forms a heterodimer with c-Fos to form the AP-1 transcription factor, which is involved in cell proliferation and activation. c-Jun mRNA is upregulated upon macrophage differentiation of bipotential myeloid cell lines, and stable transfection of c-Jun in myeloid cell lines results in monocytic differentiation. c-Jun can physically interact with PU.1 in vitro, and studies demonstrate that c-Jun could synergise with Ets factors in inducing expression of target genes. These studies all indicate a role for c-Jun in normal myeloid development. C-Jun can be activated by the Ras pathway through phosphorylation of c-Jun by Jun kinase 1 (JNK1). In addition, c-Jun mRNA

Table 2. Concepts of myeloid development.

<table>
<thead>
<tr>
<th>Concept</th>
<th>Example</th>
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<tr>
<td>Transcription factors direct differentiation</td>
<td>GATA-1, PU.1, C/EBPα</td>
</tr>
<tr>
<td>Low level expression of multiple lineage specific transcription factors in stem cells</td>
<td>GATA-1, PU.1</td>
</tr>
<tr>
<td>Autoregulation of expression</td>
<td>GATA-1, PU.1, C/EBPα</td>
</tr>
<tr>
<td>Activation of growth factor receptors</td>
<td>GATA-1→EpoR; PU.1→GM-CSFr; C/EBPα→G-CSFr</td>
</tr>
<tr>
<td>Inhibition of alternate pathways</td>
<td>GATA inhibits PU.1</td>
</tr>
<tr>
<td>Protein-protein interactions modulate function of lineage determining factors</td>
<td>PU.1 and GATA, c-jun, AML1, C/EBP</td>
</tr>
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Figure 2. A small protein-protein interaction domain of PU.1 mediates interactions with other important haematopoietic regulators. The figure is a schematic of the PU.1 protein structure, including the amino terminal transactivation domain (TAD), PEST domain, and the Ets DNA binding domain (DBD), including the winged helix-turn-helix (wHTH) structure. 49,50 Functional domains are designated according to amino acid residue. A relatively small region of the PU.1 Ets domain mediates inhibitory interactions with GATA-2 and GATA-1, as well as cooperative and/or synergistic positive interactions with AML1 and C/EBP factors. 14,22 c-Jun acts as an important co-activator of PU.1 function. 17,22

Figure 3. PU.1 and GATA-1 inhibit each other’s function through protein-protein interaction by different mechanisms. A) The upper panel shows a model which hypothesises that in stem cells, GATA-2 blocks PU.1 and c-Jun interaction, and therefore inhibits PU.1 activation of its downstream target genes. In erythroblasts, upregulation of GATA-1 blocks coactivation of PU.1 by c-Jun. But in developing myeloid progenitors, with decreased expression of GATA-1 and GATA-2, PU.1 and c-Jun synergistically activate PU.1 target genes such as the M-CSF receptor 17. G-1: GATA-1; G-2: GATA-2. B) The bottom panel shows that PU.1 blocks GATA-1 function by inhibiting GATA-1 binding to DNA.
increases with macrophage differentiation. C-Jun expression is autoregulatory, and recent reports have shown that phosphorylation of the transcription factor MEF2C by LPS activation of p38 MAP kinase in monocyes can increase c-Jun gene transcription. A more recent report has also linked MEF2 to c-jun expression by a JNK-independent but G-protein dependent pathway.19 Finally, c-Jun has been shown to be differentially upregulated during monocytic but not granulocytic differentiation of bipotential myeloid cell lines, implying a specific role for c-Jun expression in macrophage development. These studies indicate that regulation of c-jun expression, as well as its activation, may be important in its role in myeloid development.

As noted above, Ras activation is probably important in signalling c-jun expression in monocytic cells. Ras proteins are GTP-dependent molecular switches which are essential for cell growth and differentiation. In particular, macrophage differentiation and M-CSF dependent survival are altered in transgenic mice which are essential for cell growth and differentiation. Ras proteins are GTP-dependent molecular switches that are involved in signalling c-Jun expression in monocytic cells.

Ras proteins are GTP-dependent molecular switches which are essential for cell growth and differentiation. In particular, macrophage differentiation and M-CSF dependent survival are altered in transgenic mice which express dominant suppressors of Ras signalling.20,21 Ras also serves to augment c-Jun expression. A number of haematopoietic cell lines undergo spontaneous monocytic differentiation in response to activated Ras expression,21 and M-CSF, GM-CSF, or interleukin-3 induced monocypoiosis of CD34+ cells is inhibited by N-Ras antisense oligonucleotides. Activating Ras mutations occur in 20-40% of AMI and myelodysplastic syndromes (MDS), especially chronic myelomonocytic leukemia (CML). Some patients with juvenile chronic myelogenous leukaemia (JCM) show loss of the neurofibromatosis type I (NF1) gene, a Ras GTPase activating protein, and NF1 gene loss by itself is sufficient to produce the myeloproliferative symptoms associated with human JCM. In chronic myelogenous leukemia (CML), the bcr-abl protein constitutively activates Ras and requires Ras and c-jun for transformation. Thus, Ras plays a critical role in normal myeloid differentiation and leukemogenesis.

Role of c-jun in mediating the effects of Ras on PU.1: c-jun is a JNK independent co-activator of PU.1 transcriptional activation.

One candidate mediator of Ras stimulation of PU.1 function is c-jun, whose expression can be induced and whose function can be activated by Ras. In addition, as noted above, c-jun has been implicated in monocytic differentiation. We observed no activation of PU.1 by Ras in c-jun deficient F9 cells, and activation was restored by addition of c-jun.17 A dominant negative mutant of c-jun without the basic domain blocks the activation of PU.1 by Ras in CV-1 cells (which contain c-jun), and co-transfection of M- EK1, which induces the expression of c-jun via the JNK pathway, enhances PU.1 transactivation function as much as Ras does. However, c-jun post-translational modification by Ras activation is not critical for PU.1 activation, because c-jun mutated in the JNK phosphorylation sites (serines 63 and 73) synergises with PU.1 as well as wild type c-jun does; instead, it appears that Ras activation mediates up-regulation of c-jun expression. These findings are consistent with recent results indicating that a mouse with a knock-in of the Ser63Ala/Ser73Ala mutations into both c-jun alleles develops normally, with apparently normal myeloid development, and that disruption of JNK1 does not affect monocytic development. c-jun physically interacted in vitro to the PU.1 DNA binding domain, but c-Fos did not.17 Expression of c-Fos, which heterodimerises with c-jun to form the DNA binding protein AP-1, blocked the synergy between c-jun and PU.1. It appears that c-jun, whose basic domain can bind to the PU.1 DNA binding domain directly, augments PU.1 function without binding to DNA itself, as c-jun does not bind to the M-CSF receptor promoter or the minimal promoter containing only PU.1 sites. These data suggest that Ras enhances via induction of c-jun expression the stability of PU.1 to activate critical monocytic target genes such as the M-CSF receptor promoter.17 Similar results from another laboratory have implicated synergism through PU.1, C/EBPβ, and c-jun, in which c-jun acts as a co-activator.22 A major conclusion of these experiments is that c-jun is critical for PU.1 function in transactivation of monocytic target promoters, and that c-jun acts as a specific coactivator for PU.1. In addition, the effect of Ras activation is not mediated through pathways dependent of JNK phosphorylation of c-jun, but rather through pathways which induce upregulation of c-jun expression in monocytic differentiation. Therefore, understanding c-jun expression in monocytic cells is likely to be important.

What is the role of PU.1 in stem cell and myeloid development?

More lessons from the knockouts: a major controversy regarding the role of PU.1 in haematopoiesis arose when the first groups reported on studies involving targeted disruption in mice.22,24 One mouse model was embryonic lethal and the PU.1 defect initially characterised as being a stem cell defect involving all lineages other than erythrocytes and megakaryocytes.23 In a second model, the animals were viable at birth, although they died within days to weeks.24 In these animals, the deficit was characterised as primarily involving monocytes and B-cells, with delayed maturation of T-cells and neutrophils. Several explanations were discussed for these differences. As the second model involved an insertional strategy rather than a disruption, the possibility existed that perhaps it was really a hypomorph. However, this is clearly not the case, as subsequent studies have revealed that there is no PU.1 protein detectable in the knockout cells,25 and there is no significant expression of PU.1 mRNA in the PU.1-/- foetal livers from these mice, even using a probe specific for the 5' portion of the murine PU.1 cDNA26 on long exposure of heavily loaded Northern blots, indicating that the mRNA, if transcribed, is not sufficiently stable to be detectable at significant levels.27 However, more recent studies make it appear that the two models are fairly similar in terms of what they tell us about PU.1 function in myeloid development. While the cause of the embryonic lethality in the initial model is still not understood, it is clear that in this model early myeloid cells are produced, but in the absence of PU.1 they cannot differentiate further.28,29 In one of these studies,29 introduction of PU.1 but
not CSF receptors (which are deficient in PU.1-/- cells, see below) was capable of rescuing monocyctic development, further demonstrating that it is the transcription factor PU.1 and not CSF receptors which induce differentiation. Similar studies will be discussed below for C/EBPα. Therefore, one interpretation of these data is that the differences in the mouse models may be due to strain differences or other factors which do not reflect PU.1 function. Both models support the idea that very early myeloid progenitors can develop in the absence of PU.1, but that PU.1 is important for further development, and essential for monocyte and macrophage differentiation from these earlier precursors.

Role of PU.1 in regulation of the myeloid growth factor receptors: GM-CSF receptor α, G-CSF receptor, and M-CSF receptor

Another controversy regarding PU.1 function was its role in regulation of myeloid CSF receptors. Initial transient transfection studies had identified PU.1 sites in the promoters for all three myeloid CSF receptors, those for GM-, G-, and M-CSF. However, initial reports regarding the expression of these receptors in PU.1-/- cells indicated that expression of mRNA encoding the earlier GM-CSF receptor α and G-CSF receptor were not affected in PU.1-/- cells, but only expression of M-CSF receptor mRNA was decreased. However, several recent studies have reassessed this question, and it is clear that in both PU.1 knockout models there are deficiencies of all three receptors in PU.1-/- cells. Cells from both models of PU.1 knockouts have undetectable levels of all three receptors on their surface by CSF assay, which is likely the most sensitive assay of receptor expression. In addition, direct measurement of mRNA on Northern blots of PU.1 knockout foetal livers shows a significant decrease in all three receptors (GM-, G-, and M-CSF) as well. Therefore, it is likely that the in vivo studies confirm the in vitro studies indicating that PU.1 is critical for expression of multiple CSF receptors. These results are quite distinct from those involving C/EBPα, in which C/EBPα can regulate all three in vitro, but only G-CSF receptor in the C/EBPα-/- animals in vivo (see below).

The C/EBPα: the granulocytic switch

It has become clear that induction of C/EBPα expression and/or function is an important event in the decision of a multipotential myeloid progenitor to become a granulocyte. The first inkling regarding this was studies involving its differential regulation in myeloid cells lines, suggesting that it might play a role in myelopoiesis similar to that in liver and adipocyte development. The second clue was its role in regulation of CSF receptor promoters, particularly that for G-CSF receptor. However, partly because of the redundancy of the binding and activation of C/EBP proteins to myeloid targets, it was not until analysis of C/EBPα knockout mice that it was clear that C/EBPα, and not other C/EBP proteins, was critical for early granulocyte development, and that in its absence granulocytes (and not monocytes) were blocked at an early stage of development. No phenotype was observed in heterozygote animals. The block was very selective, only granulocytes (neutrophils and eosinophils) were affected, and all other blood cell elements appeared to be normal. This is consistent with the specific upregulation of expression of C/EBPα in early granulocytic cells, and can be explained by the ability of C/EBPα to regulate the expression of multiple myeloid CSF receptors.

Transcription factors and myeloid development

Figure 4. Transcription factors and myeloid development. In this model, PU.1 is important for inducing multipotential haematopoietic cells toward myeloid multipotential progenitors, and induces monocyctic differentiation as the "default" pathway along with its co-activator, c-Jun. C/EBPα acts as a switch to induce granulocytic differentiation. Granulocyte maturation is sequentially induced by C/EBPβα early, which is critical for primary granule protein gene expression, and subsequently by C/EBPε, which is essential for secondary granule protein genes and terminal maturation. Expression of PU.1 itself may be positively regulated by C/EBPα. Mutations in C/EBPα would be expected to lead to AML, while mutations in C/EBPε might be expected to lead to secondary granule deficiency syndromes.
downregulation in monocytic cells. Other interesting aspects of the phenotype as originally described included a selective loss of CFU-G (and not other CFU, such as CFU-GM) in the mice and selective loss of responsiveness to G-CSF, due to selective loss of G-CSF receptor expression (and not GM-CSF receptor a or c- CSF receptor, in contrast to the PU.1-/- mice). Transplantation experiments demonstrated that the defect was intrinsic to the haematopoietic system.

Subsequently, it was demonstrated that these mice also have a defect in response to IL-6, and lack CFU-IL6 and expression of the IL-6 receptor-α (but not gp130, the β chain). These findings are consistent with observations that G-CSF and IL-6 may play overlapping roles in granulopoiesis, consistent with similarities in their activation of signal transduction pathways, such as the STATs. G-CSF receptor and IL-6 receptor are not likely to be the only targets of C/EBPα critical for granulocytic differentiation. Targeted disruption of the murine G-CSF receptor results in an eruption of the murine G-CSF receptor results in an phenotype. An important recently described C/EBPα target gene is c-myc. Finally, a very recent study has shown that at least in murine 32D/myelomonocytic cell lines capable of granulocytic differentiation, other genes must be disrupted to observe the C/EBPα phenotype. An important recently described C/EBPα target gene in these cells is c-myc. Finally, a very recent study has shown that at least in murine 32D cells, C/EBPα can upregulate PU.1 mRNA, suggesting that PU.1 itself might be a C/EBPα target gene in granulocytic cells.

In addition to investigating the effect of disruption of C/EBPα on myelopoiesis, other studies have shown that conditional introduction of C/EBPα into myeloid cell lines capable of granulocytic differentiation can induce genetic changes and morphologic differentiation of the cells. Remarkably, in human cells only a maximum of 48 hours of exposure to increased C/EBPα protein was needed to induce differentiation which occurred two weeks later. In addition, no more than a 3-fold increase in C/EBPα protein could induce full differentiation, which indicates that in contrast to many other types of genes, relatively small changes in lineage determining transcription factors can induce major phenotypic changes, and furthermore the phenotypic effect is much more susceptible to influences (either positive or negative) mediated by protein-protein interactions. For example if PU.1 is upregulated 5 fold with myeloid maturation, and if GATA-1 expression can downregulate PU.1 transactivation potential 4 fold, then the combination of upregulation of PU.1 expression and loss of GATA-1 inhibition would result in a net increase in PU.1 activity of 20 fold. Interestingly, although expression of C/EBPα induced morphologic differentiation of U937 cells in 17 days, gene targets of C/EBPα, including the G-CSF receptor and lactoferrin were induced within a few days. Finally, expression of C/EBPα could block monocytic differentiation, but only if C/EBPα had been expressed for a week prior to adding monocytic inducing agents. These results are consistent with a model in which the default pathway of bipotential myeloid cells is the monocytic lineage, and that in this system C/EBPα must be expressed for several days to act as a switch to change this program.

In summary, these studies showed that C/EBPα is both necessary and sufficient for induction of granulocytic differentiation. Similar results have been obtained using conditional C/EBPα constructs in murine 32D granulocytic lines, and in these cells, C/EBPα, but not PU.1, could induce granulopoietic development.

Role of C/EBPα in human leukaemia (Figure 5) While this chapter is focused on normal myeloid differentiation and not acute myeloid leukaemia (AML), in which myeloid maturation is blocked, studying defects in AML will be useful in understanding normal differentiation. Clearly, regulators such as PU.1 and C/EBPα, which are critical for myeloid development and whose disruption results in a state in

C/EBPα and AML
(adapted from Look, Science 278, 1059, 1997)

![Figure 5. Abnormalities of C/EBPα in 3 different subtypes of AML. The circled subtypes represent the subtypes of AML patients in which abnormalities in C/EBPα function have been demonstrated. This includes FAB M2 with t(8;21), in which C/EBPα function is inhibited; M2 with a normal karyotype, in which approximately 25% of patients have a dominant negative mutation in C/EBPα; and M3 (acute promyelocytic), in which t(15;17) PML/RARα fusion protein inhibits C/EBPα DNA binding.](image-url)
which myeloid differentiation is blocked, are ideal candidate target genes for abnormalities in AM L, including mutations, decreased expression, and abrogation of function by translocation proteins. Recent studies have implicated C/EBPβ as a target for at least two translocation proteins which account for a significant percentage of AM L cases. AM L/ETO, the t(8;21) AML fusion protein, has recently been shown to inhibit C/EBPβ transactivation function, perhaps by recruiting co-repressor molecules through the ETO part of the fusion protein. Secondly, the PM L/RARα fusion protein found in t(15;17) acute promyelocytic leukaemia (APL) can inhibit the DNA binding activity of C/EBPβ in an all-trans retinoic acid dependent manner. These studies suggest that C/EBPβ is a major target for retinoi signalling in normal myelopoiesis, and this transcription factor may be the major target for the PM L/RARα fusion protein in APL. Finally, a screen of a large number of AML patients revealed the presence of C/EBPβ mutations in a single subtype of AML: FAB M2 patients without other cytogenetic abnormalities, and in particular no t(8;21). Based on our studies of normal myelopoiesis, we predict that PU.1 AM L will be found, perhaps in either early (M0, M1) or monocytic (M4) leukaemias.

C/EBPβ: a novel C/EBP protein essential for terminal myeloid maturation

Although the rat equivalent of C/EBPβ had been cloned several years ago, its role in myeloid maturation was not apparent until two groups cloned the human gene. Targeted disruption of C/EBPβ results in a myeloid phenotype which is quite distinct from that of C/EBPα. Disruption of C/EBPβ results in a viable and fertile mouse whose major phenotype is a block at the terminal maturation stage (metamyelocyte) of granulocyte differentiation (Figure 4). The granulocytes of the mice have a number of functional abnormalities which inhibit their ability to kill bacterial targets. Genetically, in contrast to the C/EBPα mice, in which both primary and secondary neutrophil granule protein mRNAs are absent, in C/EBPβ−/− mice primary granule mRNA is comparable to wild type, and there is selective deficiency in secondary granule protein mRNA production. Therefore, knockout studies have resulted in illuminating the fascinating use of two C/EBP proteins at different stages of granulocyte maturation: C/EBPβ appears to act early, prior to primary granule gene expression, and C/EBPα acts much later, at the time of secondary granule gene transcription (Figure 4). Consistent with a role for C/EBPβ in early myeloid development and C/EBPα acting later, a fairly large study of AM L and myelodysplastic (MDS) patients found no mutations or rearrangements in the C/EBPβ gene. More in keeping with the C/EBPβ knockout studies is a report that at least one secondary granule deficiency patient has what appears to be a functionally significant mutation in both C/EBPβ alleles.

Issues for future study

What are the major areas and questions involving myeloid development? Clearly, the control of expression and function of regulators such as PU.1 and C/EBPα is critical, and we know very little about this subject. We know very little about feedback mechanisms, although a passive model, in which receptors serve to control CSF levels, probably operates in megakaryocytopenias. If such a mechanism were operative in myelopoiesis, then in C/EBPα−/− mice, which have undetectable levels of G-CSF receptors, we would predict finding elevated G-CSF levels; we are currently testing this hypothesis. Another important area for investigation is further identification of protein-protein interaction partners, whose importance was discussed at the beginning of this chapter. Clearly, identification of the mechanism of action and gene targets for myeloid homeobox genes will be important. And finally, a very important area of investigation, and perhaps most relevant to medicine, is to relate our knowledge of normal human myeloid development to the pathogenesis of AM L and vice versa.

Acknowledgments

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Blood coagulation proceeds and is terminated by successive formation of macromolecular complexes

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The cascade or waterfall theories of blood coagulation (1964) envisaged a series of proteolytic activation steps, each consisting of one enzyme acting on one substrate, the substrate in turn becoming active to cleave the next zymogen in the series. Over the past three decades it has become clear that, with only two exceptions (activation of factor IX by factor Xa and cleavage of fibrinogen by thrombin), the activation steps occur upon formation of macromolecular complexes involving at least three proteins and a phospholipid surface containing negatively charged head groups. A structural picture has emerged through the application of various biochemical techniques to supplement the insights gained from enzyme kinetics. The structural studies both illuminate and are informed by the biochemical and mutation data, the latter deriving both from in vitro mutagenesis and clinical genetic studies. This short review will focus on the three major activator complexes involved in thrombin generation, as most is known of their structure and function. These are the tissue factor (TF)/factor VIIa, factor VIIIa/factor IXa and factor Va/factor Xa complexes which activate respectively factor IXa and/or factor Xa, factor Xa and prothrombin. Finally, some remarks on inhibitory complexes are appended.

The tissue factor/ factor VIIa complex

Following the crystallisation of TF and two years later the TF/VIIa complex all the biochemical data on the formation of the initiator complex could be mapped onto a high resolution structure, providing a huge advance in understanding of the first step in thrombin generation. Figure 1 is a ribbon representation of the structure of the complex based on the coordinates deposited at the Protein Data Bank (PDB). In this view the phospholipid surface is at the bottom of the picture where the transmembrane domain of TF would lie. TF is coloured cyan throughout its two domains, each of which is a β sandwich resembling the fibronectin type two domain. These are connected rigidly by an interface consisting of a large buried hydrophobic region, so that the angle between domains is about 120 degrees. Oriented side by side with TF are the GLA, EGF1 and EGF2 domains of factor VIIa. The GLA (γ-carboxy glutamic acid) domain shown in red has its hydrophobic loop pointing downwards onto or into the putative phospholipid surface. Clearly such a surface will orientate enzyme (factor VIIa) and co-factor (tissue factor) correctly for their subsequent binding to each other to occur. The EGF 1 domain (shown in green) of Factor VII makes intimate contacts with an exposed hydrophobic Phe residue on the short projecting α helical loop of TF domain 1. Further close binding contacts occur between the EGF 2 domain (plum coloured) of factor VII sits on top of the distal surface of TF domain 2. It can be seen that this arrangement of an extended binding surface provided by the relatively rigid TF serves to subdue the active site of factor VIIa at a precise height from and orientation relative to the phospholipid surface. Undoubtedly it is this mechanical and geometrical arrangement that serves to promote the rapid specific cleavage of either of two macromolecular substrates, factor X or factor IX, which plausibly fit into the space below the active site (identified by the inhibitor hetero atoms in white stick). These substrates also have GLA domains that bind to negatively charged phospholipid surfaces and closely resemble factor VII in overall domain structure and sequence. However neither binds tightly to tissue factor nor is activated by such binding. A unique feature of the interaction of tissue factor with factor VIIa is the massive enhancement of activity of the enzyme to both small and large substrates. In the case of the former this cannot be attributed to geometrical rearrangements of the whole factor VII molecule but must be due to some allosteric effect on the protease domain. Until the recent crystallisation of free factor VIIa the mechanism for this was purely conjectural. With information from the free structure a plausible case has been made that a shift in the loop below the active site enhances accessibility of the active site cleft. However in this as well as in the complexed structure the active conformation of factor VIIa is ‘forced’ by the binding of an irreversible inhibitor to the active site serine so that the active site structures of bound and free protease domains are virtually identical. Further information to illuminate this crucial function of tissue factor would come from the structure of uninhibited free factor VIIa if the technical problem of enzyme autodigestion during crystallisation could be overcome.

The factor VIIIa/ factor IXa Complex-‘Tenase’

Figure 2 is a cartoon of the overall structure of this complex based on two dimensional lipid film crystallography of factor VIII using models of the A domain based on ceruloplasmin crystallographic
structure and of the C2 domain based on the structure of galactose oxidase. The factor IX structure is from porcine factor IX crystallographic coordinates. Hence the position and orientation of the factor VIII A domains with respect to a lipid surface are backed by direct biophysical data. The orientation of the C domains is more conjectural but fits the available density map and the structure of isolated factor VIII C2 domain has now been solved, with results that fit the overall dimensions used for this model. Positioning the factor IX molecule in this cartoon utilises the position of the known surface loops of factor VIII which bind to factor IX protease and EGF2 domains respectively. Finally the cartoon allows the active site of factor IX to be positioned at the biophysically measured distance from the phospholipid surface. As in the case of the TF/VIIa complex, the substrate factor X can be proposed to bind to the same surface and be correctly oriented for proteolytic attack by IXa by further contacts with either or both factor VIIIa and factor IXa. Figure 3 shows the complex in ribbons models where the coordinates are taken from crystal structures or models of homologous domains as indicated above except that the C2 coordinates are crystallographic. Multiple lines of biochemical and biophysical data now support this overall picture of the ‘tenase complex’, but of course an actual high or medium resolution structure of this complex is much to be desired.

The factor Va/ factor Xa complex—‘prothrombinase’

Extensive biochemical data on the formation and function of the prothrombinase complex have established its crucial role in amplifying the signal from the two preceding complexes to provide an explosive burst of thrombin generation at the site of vascular injury. However only limited biophysical data are cur-
by the strong evolutionary correspondence between these two complexes. The future goals for research in this area include high-resolution structures of modules and complexes such that the atomic detail of these vital interactions can be visualised. Given recent progress in protein expression and in techniques for two and three-dimensional structural determination this goal is very probably achievable within the next few years.

Inhibitory complexes in coagulation
Several complexes are now known to play an essential role in damping thrombin generation in addition to the serpin complexes. These are the thrombin/thrombomodulin complex which activates protein C, the activated protein C/protein S complex which inactivates factors Va and VIIIa and the TFPI/Xa complex which binds to and inactivates TF/VIIa. As yet insufficient biophysical data is available to attempt modelling these structures, which remain for future research to solve.

References
3. Coordinates and models available at the factor VII database URL: http://europium.csc.mrc.ac.uk
Fibrinogen and its structural interactions

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The conversion of fibrinogen into an insoluble fibrin polymer by the protease thrombin is the central event in the formation of a blood clot. Not only must the fibrin polymer be able to assemble quickly to maintain the structural integrity of the vascular system, it must also be able to disperse easily to make way for more permanent tissue repair. The fibrin system has thus evolved, not only to self-assemble but also to interact with a host of other proteins involved in blood coagulation, fibrinolysis and cell-matrix interactions, subtly controlling its own formation and destruction. After more than a century of study, the fibrinogen molecule is finally revealing some of its secrets at the atomic level. This is largely due to the work of several laboratories involved in the X-ray crystallographic structure determination of core fragments of fibrinogen. These structures have confirmed many of the interactions known to take place in the fibrinogen/fibrin systems and have also provided new insights, enriching the molecular model of fibrin polymerisation.

Introduction

Work on fibrinogen began in the late 19th century when de Commercy and Hammarsten showed that fibrinogen was a unique protein native to blood plasma and that thrombin catalysed its conversion to fibrin. Early fibre diffraction studies by Astbury and co-workers established that fibrinogen and fibrin both had characteristic 5.1Å meridional reflections, unequivocally showing that thrombin acts not as a denaturant, as was the current belief, but as a pro- enyme modifier of fibrinogen. Subsequently, it was shown that these reflections were caused by portions of fibrinogen which were composed of coiled coils of two or three α helices. In the 1950s electron microscopy established fibrinogen as being an elongated trinodular molecule, of approximate length 475Å which when polymerised into fibrin had a repeating half stagger distance of approximately 230Å. Combined with extensive biochemical and sequence experiments (for details see Doolittle) a comprehensive biochemical/biophysical model of fibrinogen and its thrombin-mediated polymerisation was constructed.

Fibrinogen is a large glycoprotein (molecular weight 340kDa) consisting of three pairs of chains (α1β1γ1) interconnected by a set of 29 disulphide bonds. The six N-termini of the chains combine to create the central nodule and form a molecular dyad. Around this dyad two identical distal nodules are formed from the C-termini of the three unique chains. These three domains are connected by two sections of three stranded α helical coiled coils. The β and γ chains terminate in homologous globular domains whilst the α chain C-terminal differs markedly from the other two, consisting of a long stretch of repeated sequence terminating in an unrelated domain. This region of α-chain is quickly separated from the main body of fibrinogen by proteolytic enzymes at the beginning of the coagulation process.

In response to injury a complex cascade of serine proteases is initiated finally leading to the conversion of prothrombin into thrombin. Thrombin catalyses the transformation of soluble fibrinogen to fibrin by the removal of two N-terminal sequences of the α and β chains, the A and B fibrinopeptides. These newly exposed N-termini (knobs) are recognised by specific binding regions (holes) in the distal regions of the fibrin monomer. Because of the dyad present in the central domain, each fibrin monomer brings together two other fibrin monomers initiating the building of a protofibril. In concert with this, a transglutaminase, factor XIIIa, also activated by thrombin, cross-links the C-terminal regions of the A chains between neighbouring molecules and in due course residues in the exposed γ chains cross-linking strengthens the clot and allows for the development of a branched three-dimensional structure. In this way the fibrin polymer provides the scaffold for the platelet plug, binding to both platelets and endothelial cells via cell surface receptors such as integrins.

Another protease, plasmin, a multi-functional protease that cuts fibrinogen and fibrin at a number of specific cleavage points brings about dispersal of the fibrin polymer. In the laboratory, core fragments of fibrinogen can be generated with plasmin (or trypsin). These fragments, historically denoted as fragments D and E, correspond to the central and distal nodules observed in electron microscopy images respectively. Limited proteolysis of a mature fibrin polymer yields two complexes of the fragments, a covalent dimer, double-D (D-D or D-dimer) and the less stable D-E.

Structures

Although crystals of fragment X (a modified fibrinogen lacking the C-terminal α-chain residues) have been produced since the late 1970s, until recently only initial low resolution electron microscopic
images of fragment X have been produced. High resolution structures of fibrinogen fragments began to emerge recently with the 30kDa recombinant fragment of the γ/H9253 chain C-terminal domain9 closely followed by the larger 86kDa fragment D and its cross-linked counterpart from fibrin.10 Ultimately these structures have aided the completion of the much anticipated bovine fragment X molecule.11

Two homologous domains occur at the C-terminal of the γ/H9252 and γ/H9253 chains of fragment D. These domains are known to occur in a number of proteins always at the C-terminal end of the molecule and have been coined fibrinogen related domains (FReDs).12 The domains have a novel fold, first described by Yee et al.,9 and are composed of three subdomains, a small connecting subdomain, a central subdomain containing a seven stranded antiparallel β sheet flanked by a couple of α helices and a binding subdomain consisting of three extended loops. The binding subdomain, conspicuous in its lack of secondary structure, forms an extensive negatively charged pocket.

Evolution

Previous work on the homologous nature and sequence similarity with other proteins has pointed to a homotrimeric origin of the fibrinogen molecule. The structure provides even more compelling evidence that this is the case. When viewed on end, the β and γC domains form two points of a pseudo-three fold axis, the structures being approximately 120 degrees apart;14 rotation of either of these domains fills the gap almost perfectly (Figure 2). Interestingly, the observed arrangement of the three inter-chain disulphide bonds located at the neck of the structure differs from the symmetric assembly initially proposed.15 This topology facilitates the folding back of the α-chain C-terminal α helix and suggests that the disulphide arrangement has either changed or ap-
peared as the individual chains diverged.

A domain homologous to the β and γC-terminal domains does exist and was initially found in minor forms of fibrinogen in chickens and lampreys. In humans, this domain (denoted αEC) occurs at the C-terminal end of normal fibrinogen in a variant form known as fibrinogen-420. Because this domain occurs after the fold back of the C-terminal of α-chain it could not occur at the pseudo-threefold hypothesised above.

A recombinant form of this αEC domain from human fibrinogen-420 has been crystallised and the structure determined at 2.1 Å resolution. The structure is very similar to that of the β and γ chain domains having a root mean square (r.m.s) deviation between aligned Cα atoms of 1.26 and 0.96 Å respectively. The binding cleft exists in the αEC domain but is reduced with respect to β and γ and does not appear to bind peptides. Other FReDs have been found at the C terminals of numerous proteins, some of which have been shown to be lectins. It is possible that the αEC domain serves a similar role and binds certain carbohydrate moieties, although experimental evidence is lacking.

The D-D interface

As has been pointed out, formation of a protofibril involves the bringing together of two fragment D domains onto one fragment E domain. Cross-linking with factor XIIIa covalently associates these fibrin monomers which when proteolysed with plasmin or trypsin produces the entity double-D or D-dimer. The crystal structure of double-D thus reveals the interface between the two fibrin monomers in the blood clot. The abutment, which incidentally is the same as that observed in crystals of uncross-linked fragment D, occurs between the two γC-domains and precludes an area of 750 Å² upon formation. Binding between the two γC-domains is weak with no obvious electrostatic interactions and only a few hydrogen bonds available. The crevice appears to be occupied by a significant amount of free solvent (Figure 3a). Although fragment D has a proclivity to form this interface even in the absence of cross-linking it does seem that the clot is strengthened considerably by fragment E straddling the two molecules.

The most interesting observation about the interface is that unlike most homo-oligomeric molecular interactions the D-D interface is not symmetric and is actually offset by a translation of 13 Å about the two-fold axis. For example, a key residue in the formation of the interface, Argγ275, has two different partners, interacting with Tyrγ280 in one direction and Serγ300 in the other (Figure 3a).

On one side of the interface, both C-termini of the
abutting γC-domains can present themselves for cross-linking or binding to integrin cell surface rece-
tors (Figure 3a), whilst on the other the two γ-
binding holes are accessible to the fragment E knobs (Figure 3c). In the absence of the fragment E structure it was simple to build a low resolution model of the D2E fragment using the surrogate α-knobs, the two fold axis and knowledge of sequence as con-
straints for the representation10 (Figure 3c). Because the D2E fragment is the basic building block of the fibrin polymer simple transformations could be used to build up a hypothetical protofibril obeying the observed 230Å half stagger distance and electron mi-
oscopic images (Figure 3d).

Interaction with fragment E

Peptides containing similar sequences to those of the α and β chain knobs bind to fibrinogen mimicking and inhibiting the interaction of fragment D with fragment E.21 These sequences containing the residues GPRPam and GHRPam imitate the α and β chain knobs respectively and have very similar structures (Figure 4). Four structures have been solved containing all possible pairs of peptides i.e. GPRPam or GHRPam singly, both GPRPam and GHRPam, or none.10,22,23 The basic interaction between the ligand and binding hole appears to be primarily electrosta-
tic, positively charged arginine and the α-amino group of the peptide attracting the negatively charged binding hole.10,22 The two binding pockets of the β and γC domains do exhibit differential specificity for the two peptides, the γ-hole binding both GPRPam and GHRPam if present singly but predominantly binding GPRPam in the presence of both ligands. The β-hole does not bind GPRPam, but does bind GHRPam.22,23 Although chemically, this specificity is puzzling, as the histidine seems to have little struc-
tural interaction with residues in the binding pocket 22, biologically it makes sense. The α-chain knob contributes primarily to the formation of protofibrils and is released by thrombin first, the β-chain knob, released after a lag, helps to thicken the fibre laterally via interactions with the β-hole. Of course these peptides are merely four residue entities and do not have the remaining fragment E connected. The im-
iminent release of the structure of fragment X may help to clarify this situation.11

Cross-linking

Electron density maps calculated from four differ-
ent crystal forms of reciprocally cross-linked (between residues Glnγ398 and Lysγ406) fragment D, have shown little electron density for residues beyond γ394, although biochemical analysis of the crystals has shown the fragments to be covalently linked. This is disappointing as the C-terminal region of the γ-
chain is of much biochemical interest, containing the binding site for the platelet integrin αIIbβ3 24 and the binding region of factor XIIa as well as a ligand for the clumping receptor on pathogenic staphylococ-
ci.25 However, X-ray structures of the peptide γ398-
γ411 attached to the carrier proteins lysozyme and glutathione-S-transferase (GST)26,27 and structures of recombinant γ-domains9 have shown that the structure of this peptide is highly flexible; perhaps consist-
tent with its multiple biological roles.

During the formation of a protofibril two different schemes of cross-linking by factor XIIa can theoretically take place, one in which the cross-linking is end-
to-end (longitudinal) and one in which cross-links are created across the protofibril (transverse). The crystal structures strongly favour the former; although electron density for the cross-link is not visi-
able, simple Euclidean distance arguments do not al-
low a cross-link to any other symmetry related mole-
cule except that of the opposing end-to-end mole-
cule24,25 (Figure 3a and 3d). Of course, the fibrin net-
work consists of branching networks of fibres and therefore does not eliminate the formation of side-to-
side cross-linking where distance constraints allow. It does however eliminate this arrangement in the stan-
dard protofibril model of fibrin polymer formation (Figure 3d).

Interaction with calcium

It is well known that calcium is important in the co-
agulation process, and that fibrinogen has three high affinity binding site for calcium and an indeterminate number of low affinity sites. One high affinity sites is located in γC-domain of fibrinogen terminus,8 with a homologous calcium existing in the βC-domain.22 Two other calciums have been shown to exist in structures of fragment D; one located in a pocket between the coiled coil and the βC-domain the other in the γC-domain. Of the two homologous calciums the βC-domain associated ion has a weaker binding affinity than that of the γC-domain, primarily be-
cause of a five-residue insertion eliminating one of the co-ordination sites. These calciums appears to contribute little to the binding of peptides or fragment E and seem to be responsible for maintaining the structural integrity of the domain; indeed the γC-
domain is more vulnerable to plasmin degradation in the absence of calcium. The presence of homologous calciums in each of the structures of FrEd domains so far elucidated,10,20 points to the necessity of this calcium site in the fold.

The second site in the γC-domain occurs as a re-
sult of binding of the GHRPam ligand to the γ hole
and is mainly a consequence of a small shift in the polypeptide backbone around the residues Gly296-Asp297.29 The remaining site is more interesting and appears to influence a conformational change controlling the formation of a correct binding pocket for the B-chain knob. In the absence of GHRPam a calcium ion is co-ordinated between charged residues in the coiled coil (Glu132) and β-domain (Asp261 and Gly263). The presence of GHRPam causes the loop containing residues β261-263 to flip into the correct position to form a binding pocket thus removing the calcium.23 This effect is undoubtedly subtle and its role in fibrin polymer formation still remains to be fully explored.

Conclusions and future prospects

The solution of several core fragments of fibrinogen and their counterparts from fibrin has proved invaluable in the search for a complete molecular model of fibrin polymerisation. The current round of structures will culminate in the soon to be released structure of bovine fibrinogen fragment X which should provide structural information on fragment E, missing from previous studies. This work, however, should only be regarded as a beginning and many more experiments need to be performed to gain insight into structural interactions of the fibrin/fibrin-ogen system. Higher resolution studies and the structures of intact fibrinogen molecule all should ensue. Not only this, but the D,E complex, the basic building block of the fibrin clot, and structures of complexes with other proteins such as factor XIII, t-PA and integrin αIIβ remain to be elucidated.

Thrombosis and haemorrhage present major medical problems for a large number of people. A detailed knowledge of the structures of the proteins involved in their pathogenesis has already begun to aid our interpretation of these disorders.28,29 Further studies will not only supplement our knowledge of this fascinating system but may also aid in the treatment of such haemostatic irregularities.

Note

The structures mentioned in the text are all available in the PDB database available at http://www.rcsb.org. Codes 1FZA, 1FZB, 1FZC, 1FZE, 1FZF and 1FZG are the structures of fragment D of human fibrinogen and its cross-linked counterpart from fibrin. Nature 1997; 39:455-62.


References


Antithrombin and its endothelial modulation

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A prime lesson from research over the last decade is that coagulation is a surface-based process. The interaction of individual coagulation factors with the cells lining the microvasculature, or with circulating phospholipid complexes modulates their activities and can even switch their functions from a coagulative to an anticoagulative role. Thus whilst the pathways of extrinsic and intrinsic coagulation that we all know from text books are still relevant, their simplified form bears as much resemblance to reality as a stone age nose-flute does to a modern clarinet. In each case evolution has changed a basic process into one of much greater subtlety and versatility. The extraordinary complexity in the function of just a single clotting factor is illustrated here, in structural detail, with antithrombin the principal inhibitor of the main coagulation proteases.

Antithrombin acts at two protective levels: firstly, as a low activity inhibitor in the large arterial circulation and, secondly, as a highly active inhibitor in the more sluggish circulation of the capillary beds and their venous outflow. The difference is primarily due to the binding and activation of antithrombin by the heparan glycosaminoglycans of the microcirculation. But even this process is self-modulatory as the two different antithrombin components of the plasma, α-antithrombin with 4 carbohydrate side-chains and β-antithrombin with 3, bind to the heparans with different affinities and hence differ in the localisation of their activities differs in the circulation. To complicate matters further, it is now becoming apparent that the inactivated products of antithrombin, and in particular its L-(latent)-configuration, may modify the function of the remaining active component and act as a signal for diverse cellular responses.

Structures of antithrombin
Antithrombin is now one of the best studied members of the serpin family of serine protease inhibitors, with the completion of the structures of some 9 different conformations of the molecule (Figure 1). The first structure of antithrombin discovered was that of its cleaved form, described by Mourey et al. in 1993, but the real surprise came with the structure of the intact active form of antithrombin determined in 1994 in Gottingen and Cambridge. These structures of active antithrombin each showed the hinge of the peptide loop containing the reactive centre (P1) argininetobe partiallyinserted into the main β-sheet of the molecule. This constrains the movement of the loop in a conformation that would predictably have low activity as a protease inhibitor. This was borne out by subsequent crystallisation of the complex of antithrombin with the core heparan/heparin pentasaccharide, determined by a graduate student, Lei Jin. This structure showed the expulsion of the reactive loop from the sheet into a free conformation as in the active forms of other serpins. But there was also another outcome from this work; Lei Jin showed that mixing equimolar proportions of antithrombin with pre-formed latent-antithrombin gave consistent crystallisation of the mixed heterodimer. Both the Dutch and the Cambridge groups had previously found by trial and error that active antithrombin crystallised as a heterodimer, which both groups now agree is due to linkage of the active and latent forms of antithrombin. The significance of this formation of latent antithrombin in crystallisation solutions was not immediately apparent, but in retrospect this was the first evidence that antithrombin could undergo a spontaneous transition from its active 5-stranded A-sheet form to the inactive 6-stranded latent form. The bonus from these observations has been the ability to crystallise a series of structures of antithrombin, including β- as well as α-antithrombin, in various conformations and in complexes with peptides and oligosaccharides. Our colleagues James Whisstock and Arthur Lesk have carried out comparisons of these structures to deduce the conformational changes involved in activation. The mechanism involved has been further clarified by Airlie McCoy, by the most recently demonstrated structure of a complex of antithrombin with fluorescein, which shows the molecule in an intermediate activated conformation.

Heparan binding
Antithrombin binds avidly to a specific pentasaccharide fragment present in the heparans of the microvasculature but which is best known to us because of its presence in the animal-derived anticoagulant heparin. It is this pentasaccharide that is the essential active component of the low molecular weight heparins. Heparin itself is not a physiological species but is a hydrolysate of animal heparans, hence the heterogeneity of current therapeutic products. Even in...
the most refined of heparins, only one third of the oligosaccharide chains present contain the core pentasaccharide essential for their anticoagulant function. In the circulation, the binding of antithrombin normally occurs to the pentasaccharide core of the heparans that line the microvasculature and its subendothelial spaces. Binding takes place between negatively charged sulphate groups of the pentasaccharide and a basic site on the flank of the antithrombin molecule extending from the middle of the D helix (Arg 129, Lys 125) to the base of the A helix (Arg 46, 47), and to a new induced helical extension (Lys 114). The pentasaccharide fragment fits within, and bonds to, the amino-terminus extension of antithrombin (Lys 11, Arg 13).

Mechanism of activation, inhibition and release

The structure of the complex of activated antithrombin with the pentasaccharide clearly demonstrates the changes that result in its activation as an inhibitor, with release of the reactive centre peptide loop of antithrombin to give a conformation that acts as a bait - or ideal substrate - for the coagulation protease thrombin and factor Xa. It was earlier proposed by van Boekel that this activation was promulgated by an extension of the D helix and indeed as predicted there is an increase of two turns in the helix consequent to the binding of the heparan/heparin pentasaccharide and a basic site on the flank of the antithrombin molecule extending from the middle of the D helix (Arg 129, Lys 125) to the base of the A helix (Arg 46, 47), and to a new induced helical extension (Lys 114). The pentasaccharide fragment fits within, and bonds to, the amino-terminus extension of antithrombin (Lys 11, Arg 13).

The activation of antithrombin on binding to the pentasaccharide core of heparan is only part of the story. The effective inhibition of thrombin, but not factor Xa, also requires an extension of the polysaccharide chain for some 12 units beyond the pentasaccharide core. This allows a bridging stabilisation of the antithrombin-thrombin complex by further linkage of the heparan/heparin to an anion binding site on thrombin. Moreover, the complexation of the coagulation proteases is quickly followed by the cleavage of antithrombin with insertion of the reactive loop into the A-sheet of the molecule. As indicated diagrammatically in Figure 2, the protease moves to the opposite pole of the molecule and concomitant with this the heparin binding site reverts to a low-affinity conformation. This results in the release of the complex back into the circulation for catabolic removal.
Conformational inactivation and the onset of thrombosis

The inhibitory function of the serpins is dependent on a profound conformational change in which the peptide loop containing their reactive centre (P1) residue springs back into the body of the molecule to irreversibly bind the target protease. In this mousetrap-like action the exposed 14-residue peptide loop (P14-P1) is buried by insertion as an extra strand in the A β-sheet of the molecule. This sophisticated method of entrapment has also allowed evolution to modify the mechanism to modulate the action of individual inhibitors. In this way PAI-1 has a desirably brief half-life as it spontaneously inactivates itself by insertion of its reactive loop in the A β-sheet to give latent PAI-1.

Antithrombin has recently been shown to undergo a similar, though much slower, spontaneous conversion to its inactive latent conformation, with readily discernible amounts present in plasma on incubation at 37°C for 72 hours. More rapid conversion occurs on incubation of isolated antithrombin at 41°C or 50°C but the appearance on electrophoresis of free latent antithrombin is preceded by the formation, in reciprocal proportions, of a new slow band. This slow component is shown to be a heterodimer of active and latent antithrombin. It can be isolated as a single stable band either by incubation of antithrombin or by mixing equimolar proportions of active and latent antithrombin under the same conditions that give overnight crystallisation of the active/latent antithrombin heterodimer. Similarly, equimolar addition of latent antithrombin to plasma results electrophoretically in a quantitative shift to the slower heterodimer mobility. Clinically, the presence of latent antithrombin is potentially deleterious as its linkage to form the heterodimer results in inactivation of the otherwise normal molecule linked to the latent antithrombin. The additive effects of this conversion of one allele of a conformationally unstable antithrombin is illustrated in Figure 3. In the case of a-antithrombin, since the dimer readily dissociates there is only a 13% additive loss of activity, but with β-antithrombin the dimer appears more stable, with the additive loss of activity from the normal β component being 21% rising to 33% on stabilisation of the dimer with heparin. This gives a potential loss of 83% of the inhibitory activity of β-antithrombin. Such linked and selective loss of activity of β-antithrombin provides an explanation for the unexpected severity of thrombotic episodes in heterozygotes with conformationally unstable antithrombins.

The unknown messenger

Recent findings have highlighted the messenger function of spent coagulation factors. In particular animal studies have shown that PAI-1 and other serpins, subsequent to their conversion to inactive forms, act as modifiers of cell adhesion, leucotaxis, and angiogenesis. A striking but unconfirmed example was the finding of Folkman et al. that inactive 6-stranded conformations of antithrombin could inhibit angiogenesis in mice to an extent that made them effective antitumour agents. It had long been known that antithrombin could be readily induced to shift from its active 5-stranded conformation to an intact, as well as a post-cleavage, 6-stranded form. Studies of this transformation were initially confusing but it is now clear that the change can result not only in the formation of the PAI-1 like latent form, but also in a range of other polymeric forms. The proportion and nature of these various L-forms varies depending on concentration, pH and temperature. All of the L-forms however, share the same overall conformation and each has similar effects with respect to angiogenesis. The challenge ahead is to determine the resting physiological function and significance of these inactive forms of antithrombin. Thus antithrombin because of the structural knowledge of its function is acting as a prototype for the range of modulatory activities likely to be found with other coagulation factors. In particular, the findings to date are a reminder to all investigators that evolution is likely to utilise spent molecules as signals for a redeployment of cellular functions.
Acknowledgements

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References

11. Pei XY, Carrell RW, Gettins PGW, Hunting-
Iron has the capacity to accept and donate electrons readily, interconverting between ferric (Fe³⁺) and ferrous (Fe²⁺) forms. This property makes iron a useful component of cytochromes, oxygen-binding molecules (haemoglobin, myoglobin) and a variety of enzymes. However, this reactivity also leads to toxicity. Consequently, proteins sequester nearly all of the iron in the body. Iron circulates in plasma tightly bound to transferrin (Tf). Within cells iron is reversibly incorporated into ferritin. A substantial amount also exists in the form of iron protoporphyrin (heme) and in iron-sulphur clusters. Little or no free iron is found outside of these physiological sinks, though protein-bound iron can be mobilised for use and reuse.

Iron metabolism is similar among mammalian species. Over the past 80 years, several spontaneous mutations affecting iron metabolism have been described in laboratory animals. Analysis of these mutants, and discovery of the genes mutated in some of them, have greatly improved our understanding of normal iron transport processes. Examples will be cited throughout this article.

Intestinal iron absorption

Intestinal epithelial cells lining the absorptive villi close to the gastro-duodenal junction absorb iron. Absorption is facilitated by the low pH in gastric efflux, which reduces dietary iron and delivers it in a proton-rich milieu. A brush border ferric reductase aids iron reduction. Ferrous iron passes through two cellular membranes to traverse the enterocyte: the apical membrane (at the luminal surface) and the basolateral membrane (at the serosal surface). Two mouse mutants have defects affecting iron transfer across the enterocyte. These have given important insights into both apical and basolateral transport.

Mice homozygous for the microcytic anaemia (mk) mutation absorb substantially less oral iron than control animals. Isolated duodenal loop experiments have shown that mk epithelial cells take up iron poorly when it is instilled directly into the intestinal lumen. This phenotype is consistent with a defect in a critical apical iron transporter, or a protein that modulates the activity of a transporter.

Mice hemizygous or homozygous for the sex-linked anaemia (sla) mutation are also iron deficient, due to a decreased ability to absorb oral iron. Iron enters absorptive enterocytes normally, but it is not efficiently transferred across the serosal (basolateral) surface. Thus, the sla defect interferes with iron transfer at a later step than the mk defect.

Our laboratory undertook positional cloning of the mk mutation, using a backcross mapping strategy and analysis of candidate genes. We found that mk was non-recombinant with a gene designated Nramp2. Nramp2 had been identified as a homologue of the gene encoding natural resistance associated macrophage protein 1 (Nramp1), a molecule involved in defense against intracellular pathogens. Both molecules are predicted to contain 12 transmembrane domains, suggesting that they function as transporters. The mk mutation is a missense substitution in Nramp2 that introduces a charged residue into the fourth predicted transmembrane domain (gly185arg). In parallel with this discovery, Gunshin and co-workers identified Nramp2 (designated DCT1 in their report) in a Xenopus oocyte expression cloning screen for molecules capable of transporting iron. Further studies demonstrated that forced expression of wild type Nramp2 stimulated iron accumulation by mammalian cells, but expression of the mk mutant form (gly185arg) was associated with very little activity. These observations provided compelling evidence that Nramp2 is the apical iron transporter, acting at the intestinal brush border. Recently, a new name for Nramp2 was proposed, to describe its function better. Most investigators now use the term divalent metal transporter 1 (DMT1) instead of Nramp2. In addition to iron, DMT1 transports other divalent metal ions, including manganese, cobalt, copper, zinc, cadmium and lead.

The discovery of the gene mutated in sla mice provided information about the basolateral transport process. Once again, through a combined genetic mapping and candidate gene approach, Vulpe and co-workers showed that sla mice carry a mutation in a gene dubbed hephaestin, after the Greek god Hephaestus, who forged iron. The hephaestin protein is closely related to caeruloplasmin, a multicopper oxidase that can act as a ferroxidase. While it is not known precisely how hephaestin contributes to iron transfer, it seems likely that it oxidises iron as part of the transmembrane transfer process and/or the process of loading iron onto plasma transferrin. Hephaestin is not, itself, an iron transporter.

No mouse mutants have phenotypes consistent with defects in the basolateral transporter. Recently, however, a zebrafish mutant was characterised that...
has a defect in a putative iron transporter that is probably the orthologue of the mammalian basolateral transporter. This protein has been called ferroportin.

Regulation of iron absorption

The process of intestinal iron absorption is regulated in at least three ways. First, it is affected by recent dietary iron intake. This phenomenon has been referred to as mucosal block. Following a large oral iron dose, enterocytes become refractive to absorbing additional iron for several days. This probably results from accumulation of intracellular iron, leading the enterocyte to believe that its iron requirements have been met. It can occur even in the setting of systemic iron deficiency.

A second regulatory mechanism also senses available iron, but responds to total body iron, rather than dietary iron. This has been termed the stores regulator. It is capable of changing iron absorption over a two to three fold range. While the molecular mechanism of the stores regulator has not been elucidated, it probably involves sensing of the iron saturation of circulating Trf.

A third mechanism, the erythropoietic regulator, adjusts intestinal iron absorption in response to the demands of erythropoiesis, independent of body iron stores. The erythropoietic regulator involves the presence of a soluble signal from the haematopoietic bone marrow to the duodenum, but the signal has not yet been identified. It operates over a broader range than the stores regulator. The erythropoietic regulator leads to increased intestinal iron absorption in patients with thalassaemia syndromes, congenital dyserythropoietic anaemias, sideroblastic anaemias and other disorders characterised by ineffective erythropoiesis. In contrast, anaemias resulting from ineffective erythropoiesis typically do not stimulate iron absorption.

Iron trafficking

Nearly all iron circulating in the plasma of normal individuals is bound to Trf. Iron is taken into mammalian cells by receptor-mediated endocytosis of diferric Trf. Specific Trf receptors (Trfrs) on the outer face of the plasma membrane bind diferric Trf with high affinity. Trfrs are disulphide-linked homodimers of a 90 kD glycosylated polypeptide that have one transmembrane segment. The crystal structure of the extracellular portion of Trfr has recently been reported.

The short intracellular tail of the Trfr directs rapid internalisation of the Trfr-Trf complex after ligand binding, mediating invagination of clathrin-coated pits and formation of endocytic vesicles. Once internalised, endosomes are acidified to pH 5.5-6.0 through the action of an ATP-dependent proton pump. Endosomal acidification weakens binding of iron to Trf, and produces conformational changes in both Trf and Trfr, strengthening their association. Iron release may also be facilitated by a membrane-bound oxidoreductase. Iron is then exported from the endosome through a process involving DMT1.

A spontaneous mouse mutation in the gene encoding Trfr has provided a unique perspective on the Trf cycle. Hypotransferrinaemia (hpx) animals have an abnormality in splicing of Trf mRNA that results in a severe deficiency of plasma Trf. We recently showed that this results from a point mutation in a splice donor site at the beginning of the sixteenth intron of the Trf gene (Trenor et al., in preparation). Homozygous hpx mice have a severe hypochromic, microcytic anaemia, indicative of iron deficiency in the erythron, but normal development of other tissues. They die of anaemia before weaning unless treated with red cell transfusions or exogenous Trf. Although the erythron is iron deficient, a massive amount of non-Trf-bound iron is deposited in the liver and other organs of untreated neonates and treated adult animals. Most non-haematopoietic tissues ultimately show evidence of iron deposition. Homozygous hpx animals have increased intestinal iron absorption in response to their anaemia, exacerbating tissue iron deposition. Human patients deficient in Trf show similar phenotypes to hpx mice. Although not yet described in molecular detail, they are presumably to carry mutations in the Trf locus at 3q21. They have severe anaemia and develop systemic iron overload, but are otherwise normal.

These findings suggest that Trf is not universally required for efficient iron transport. The fact that non-erythroid tissues accumulate iron in the setting of Trf deficiency indicates that efficient non-Trf-bound iron transport mechanisms must exist.

Knockout mice have been produced that lack functional Trfr protein. These mice die in utero from the consequences of severe anaemia. The importance of the Trf cycle for erythropoiesis is underscored by the fact that development of most tissues appears normal prior to embryonic death. The sole exception is the primitive neuroepithelium, where loss of Trfr is associated with increased apoptotic cell death. Interestingly, there are some reports that surviving hpx mice may also have subtle neurological changes.

The difference in viability between hpx mice and Trfr knockout mice has not yet been explained. It most probably results from the increased availability of iron for non-Trf-bound iron uptake pathways in hpx mice. Trf-binding of iron may limit the amount of non-Trf cycle uptake in Trfr knockout mice.

Interestingly, heterozygous mice lacking one copy of the Trf gene demonstrate iron deficient erythropoiesis, even though they still retain one functional Trf allele. This indicates that a full complement of cell surface Trf is required for normal Fe uptake by haemoglobinising erythroid precursors.

Iron recycling

Because most of the body's iron is contained in red blood cells, recovery of iron from senescent red blood cells is essential. Only 1-2 mg of iron normally enters the body each day through the intestine, and this is balanced by iron output from shed cells and lost blood. Even under extreme circumstances, this absorption cannot be increased above about 6 mg/day. Meanwhile, the erythron has a daily requirement of 20 mg of iron. Nearly all iron for erythropoiesis is supplied through macrophage ingestion of senescent red cells and catabolism of their haemoglobin to scavange iron for reuse. The pathway iron takes with-
in the phagocytic cell and the mechanism for loading recovered iron onto circulating apo-Trf are not yet understood.

Patients with anaemia of chronic inflammation (anaemia of chronic disease) have defective recycling of iron by reticuloendothelial macrophages. This condition probably results from a change in macrophage iron handling in response to cytokine signals, but the details are not known. In any event, iron builds up in macrophages and is less available to erythroid precursors. It is also less available to pathogenic microorganisms, which almost invariably need iron for their proliferation. In this way, iron withholding may be an adaptive and advantageous response; mild anaemia is a relatively low price to pay for a chance at attenuating infection. Much remains to be learned about macrophage iron recycling.

**Iron storage**

The liver plays an important role in iron homeostasis, because it is the major depot for storage iron. Like the duodenal enterocyte, the hepatocyte is capable of iron uptake, storage and export. The iron transporters involved in uptake and release of iron from hepatocytes have not been identified. Caeruloplasmin probably acts as a ferroxidase facilitating hepatocyte iron export, but its mechanism of action is not defined.

**Human disorders of iron metabolism**

**Iron deficiency disorders**

Iron deficiency remains an important public health problem affecting up to a billion individuals worldwide. Any condition in which dietary iron intake does not meet the body's demands will result in iron deficiency. Children become iron deficient because their rapid growth outstrips the iron supply. Similarly, premenopausal women become iron deficient because they regularly experience menstrual blood loss. In much of the world, chronic parasite infections result in intestinal blood loss that outpaces dietary iron intake. Other conditions associated with blood loss, such as tumors, inflammation, infections and congenital malformations, may also cause iron deficiency.

Inherited defects in iron metabolism are fascinating but poorly understood. There are several case reports describing familial iron deficiency anemia that is unresponsive to oral iron therapy and incompletely responsive to parenteral iron therapy. The gene(s) responsible for this condition has not been identified.

**Manifestations of iron overload**

Tissue iron loading occurs in one of two characteristic patterns. When plasma iron content exceeds the iron binding capacity of Trf, parenchymal cells of the liver avidly take up iron. As iron overload progresses, cardiac myocytes, pancreatic acinar cells and other cells accumulate iron. In contrast, when iron overload results from increased catabolism of erythrocytes (e.g., in patients who receive frequent blood transfusions), iron accumulates in reticuloendothelial macrophages first, and only later deposits in parenchymal cells. Parenchymal iron loading is particularly dangerous, leading to tissue damage and fibrosis. The reticuloendothelial system is generally a safe sink for iron; macrophages keep iron sequestered and attenuate its reactivity.

**Genetic iron overload disorders**

**Hereditary haemochromatosis**

Patients with hereditary haemochromatosis chronically absorb 2-3 times more dietary iron than normal individuals. They generally remain asymptomatic until adulthood, though abnormally high iron loading of Trf can be detected by adolescence. Early symptoms of haemochromatosis are non-specific and common in adults; they include fatigue, arthralgias, depression, erectile dysfunction, and increased skin pigmentation (due to increased melanisation, rather than the iron itself). As the disease progresses, patients develop iron-induced liver inflammation that progresses to fibrosis and cirrhosis. Cardiac iron deposition leads to cellular damage and arrhythmias. Damage to endocrine tissue results in diabetes mellitus, hypopituitarism, hypogonadism and hypoparathyroidism.

The gene affected in most patients with hereditary haemochromatosis encodes an atypical HLA class I molecule designated HFE (originally HLA-H). A missense mutation that converts amino acid 282 from cysteine to tyrosine (C282Y) is highly associated with haemochromatosis. Based upon haplotype analysis, it has been proposed that the majority of patients are descended from a common Celtic ancestor who lived 60-70 generations ago.

HFE-associated hereditary haemochromatosis is probably the most prevalent monogenic genetic disease in Caucasians. Based on epidemiological data from voluntary blood donors, it has been estimated that 1 in 10 white Americans carry a C282Y allele. Patients who are homozygous for this mutation usually develop iron overload; a subset of heterozygotes may also be affected. Several other polymorphisms have been found in the gene encoding HFE. An aspartic acid for histidine substitution (H63D) is extremely prevalent, but it is not always associated with iron loading. Like other HLA class I-related proteins, HFE arrives on the cell surface as a heterodimer with β2-microglobulin. However, HFE does not contain a functional peptide binding groove. It is expressed on many cells, including duodenal crypt cells and macrophages. In the three years since it was first reported in the literature, its crystal structure has been solved, and it has been shown to form a high affinity complex with Trf. Direct physical affinity measurements indicate that HFE binding decreases the affinity of Trf for Trf. However, the consequences of the HFE/Trf association in living cells remain unclear. In spite of the wealth of information that has emerged, it is still not clear how HFE regulates iron absorption.

Not surprisingly, C282Y is a loss of function mutation. Similar to human patients, knockout mice lacking murine Hfe protein (Hfe/- mice) rapidly develop iron overload. Mice have also been engineered to carry an intact Hfe gene that has been altered to include the C282Y mutation found in human patients.
These HFE C282Y/C282Y mice also develop iron overload, though to a lesser extent than Hfe-/- mice do. The C282Y mutation disrupts an intramolecular disulfide bond, and undoubtedly alters the conformation of the protein. Mice heterozygous for either the knockout or the C282Y mutation show modest but statistically significant iron loading. Although there is no doubt about the fact that the C282Y mutation causes hemochromatosis, the broad spectrum of clinical presentations in C282Y homozygotes and heterozygotes provides strong evidence that other modifying genes must affect phenotypic expression. Environmental factors, such as diet, vitamin C intake, alcohol intake, and consumption of blockers of iron absorption (e.g. tea containing tannins, antacids, etc.) also influence the pace of iron loading.

Non-HFE hemochromatosis

While the identification of deleterious mutations in the HFE gene has simplified the diagnosis of hereditary hemochromatosis, it has also made it quite apparent that there are other, non-HFE-related iron overload conditions that are similar in clinical presentation.60-66 One subgroup of patients with non-HFE hemochromatosis is characterized by a genetic predisposition to severe iron loading over the first three decades of life. This disorder has been termed juvenile hemochromatosis. HFE has unequivocally been excluded as a candidate locus. It seems likely that HFE and the products of the genes responsible for non-HFE hemochromatosis are components of the stores and/or erythropoietic regulatory systems. However, to date these regulators are not well understood, and there are no good candidates for the non-HFE hemochromatosis genes.

African iron overload

Up to 10% of the population in some rural areas of sub-Saharan Africa have a genetic predisposition to iron overload.67 Formerly termed Bantu siderosis, African iron overload results in a pattern of iron deposition that differs from that seen in hereditary hemochromatosis.68 Hepatic Kupffer cells contain substantial amounts of iron, similar to transfusional siderosis, suggesting a defect in erythroid iron recycling. Hepatocytes also contain large amounts of iron. Liver disease is the predominant organ manifestation; cardiomyopathy and diabetes are seen less often. Interestingly, there is accumulation of splenic iron in African iron overload, associated with a generalized increase in iron in the reticuloendothelial system.69 This suggests that there is a relative block in iron egress from macrophages.

African iron overload is exacerbated by increased dietary iron intake in individuals who drink a traditional beer that is brewed in non-galvanised steel drums. At one time the condition was attributed strictly to dietary excess, but not all beer drinkers develop clinically significant iron overload, and not all iron loaded patients consume excessive amounts of the beer. It has been difficult to determine the inheritance pattern because of the prevalence of this disorder, and the influence of diet. An hypothesis has been put forth that the trait is inherited in a dominant fashion, with heterozygotes showing clinically significant iron overload.69 Presumably homozygotes are more severely affected. As would be expected based on its distinct pathology, African iron overload is not linked to the HLA locus, and is not due to mutations in the HFE gene.70,71

References

19. Lawrence CM, Ray S, Baboyonshv M, Galluser R, Borhani DW, Harrison SC. Crystal structure of the


Thalassaemia due to mutations in a transcriptional regulator, ATRX

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At all stages of development, human haemoglobin is made up of two α-like and two β-like globin chains. In embryonic life the ζ and ε genes are fully active producing embryonic haemoglobin (ζεαα). Between 6 to 8 weeks of gestation there is a switch in expression so that the α and γ globin genes become fully expressed producing foetal haemoglobin (αγββ). Finally at around the time of birth there is a further switch in the β globin cluster from γ to β globin expression so that in adult red cells haemoglobin A (αβββ) predominates.1

In α thalassaemia, the synthesis of α globin chains is downregulated so that in foetal life there is anaemia and the excess γ globin chains form soluble tetramers (γ4) called Haemoglobin Bart’s. In adult life, α thalassaemia also causes anaemia but, since by this time the γ to β switch is complete, the excess non-α chains assemble into β4 tetramers, called Haemoglobin H. The degree of anaemia and the amounts of the abnormal haemoglobins (Bart’s and H) produced broadly reflect the degree to which α globin synthesis has been downregulated.2

Normal individuals have four α globin genes, arranged as linked pairs of genes at the tip of each copy of chromosome 16, written in shorthand as αα/αα. α thalassaemia most commonly results from the inactivation of one or both α genes from chromosome 16. Carriers of α thalassaemia have a mild hypochromic microcytic anaemia and may produce detectable amounts of Hb Bart’s at birth. In addition, occasional cells containing haemoglobin H may be detected in adult carriers of α thalassaemia although haemoglobin electrophoresis is unremarkable. In all other respects carriers of α thalassaemia are normal.2

α thalassaemia is probably the most common disease-causing mutation affecting mankind. There is good circumstantial evidence that α thalassaemia has attained such high frequencies by virtue of its selective advantage in areas where Falciparum malaria is or has been endemic. Therefore, in tropical and subtropical regions of the world the carrier frequency of α thalassaemia is very high. In these areas, some individuals inherit only one functional α gene which causes considerable imbalance in the synthesis of α and β globin chains resulting in a haemolytic anaemia, with large amounts of Hb Bart’s at birth and similar levels of Hb H in adult life, a condition known as Hb H disease. Similarly, in these areas some infants inherit no functional α genes. This produces a lethal condition referred to as the Hb Bart’s hydrops foetalis syndrome.2

The molecular basis of these common forms of a thalassaemia is now understood in detail. Normally, the α-like genes are arranged along chromosome 16 in the order in which they are expressed in development (telomere-ζαγβαε-centromere, Figure 1). Furthermore, we now know that the cluster lies in a telomeric, gene-rich region of the genome, surrounded by widely expressed genes. Full expression of the α-like genes is critically dependent on the presence of a regulatory element (called HS-40) which lies 40 kb upstream of the cluster (towards the telomere). Many deletions removing one or both genes have now been characterised. In addition many different point mutations affecting the structural genes have been identified, these cause the non-deletional forms of a thalassaemia.3

In addition to these common forms of α thalassaemia, many rare and unusual molecular defects have been identified. These are important because they provide an explanation for patients with hitherto undiagnosed anaemia, but they also help us to understand how the α cluster is normally regulated in vivo. Rarely, α thalassaemia is caused by deletions that remove the α globin regulatory element (HS-40).4 In general these mutations have been observed outside of the malaria belt indicating that they are sporadic genetic events that have not been selected during evolution. These natural deletions first indicated the existence of this unexpected form of long range control of α globin expression. There are also two rare forms of α thalassaemia that are found in association with a variety of developmental abnormalities, and in particular with mental retardation (so called α thalassaemia with mental retardation, ATR syndromes). Patients in the first group have large (>1 Mb) deletions from the tip of chromosome 16 including the α globin genes (ATR-16). These usually result from chromosome truncations or translocations.5 Patients in the second group are now known to have mutations in a trans-acting factor (called ATRX) encoded on the X-chromosome (ATR-X syndrome).6 These patients have α thalassaemia with profound mental retardation, facial abnormalities and urogenital anomalies. In this case it is thought that the X-encoded factor regulates expression of many genes, the α genes being but one target. Analysis of
this rare form of α thalassaemia has revealed a novel mechanism by which α thalassaemia may occur and promises to elucidate in greater detail how the α globin cluster is normally regulated. Developments in our understanding of this syndrome form the basis for this brief review.

The clinical and haematological features of the ATR-X syndrome

More than 100 cases of the ATR-X syndrome from over 70 families have now been characterised and a definite phenotype is emerging (Table 1). In the majority of cases, the children have profound and global developmental delay. They have marked hypotonia as neonates and in early childhood, all milestones are delayed. Many do not walk until later in childhood and some are never ambulant. Almost all have no speech, frequently have only situational understanding, and are dependent for almost all activities of daily living. However, in one family with four affected male cousins, one has profound mental retardation, whereas the others have IQs of 41, 56 and 58 (in press). The basis for this marked variation is unknown but indicates that mutations in the ATRX gene may be responsible for a wider spectrum of intellectual handicap than previously thought.

In early childhood the distinctive facial features are most readily recognised: the frontal hair is often upswept, there is telecanthus, epicanthic folds, a flat nasal bridge and mid-face hypoplasia, a small triangular upturned nose with the alar nasae extending below the columella and septum. The upper lip is tented, the lower lip full and everted giving the mouth a carp-like appearance. There is a wide spectrum of associated abnormalities affecting many systems (Table 1) the most intriguing of which are the genital abnormalities seen in almost all children. These may be very mild as in undescended testes but the spectrum of abnormality extends through hypospadias, micropenis to external female genitalia with the affected children being defined as male pseudohermaphrodites.

The haematology is often surprisingly normal considering the presence of α thalassaemia. Neither the haemoglobin concentration nor mean cell haemoglobin are as severely affected as in the classical forms of α thalassaemia (Figure 2) and this perhaps reflects the different pathophysiology of the conditions. Where there is more than one affected member in a family there is frequently marked variation in the frequency of cells with HbH inclusions (Figure 2c). This suggests that the haematological picture is complicated by other, possibly genetic variables.

Evidence that the ATR-X syndrome is an X-linked condition

The five original cases described by Wilkie et al. were sporadic and, apart from them all being male, there were no immediate clues to the genetic aetiology. Somatic cell hybrids composed of mouse erythroleukaemia cell lines containing each copy of chromosome 16p13.3 is indicated by a vertical bar. Genes are shown as solid boxes, pseudogenes as open boxes and hypervariable regions as zig-zag lines. The α-globin regulatory region (HS –40) is shown as a striped box. The scale is in kilobases as indicated above. The haemoglobins synthesised during development are shown below.

Table 1. Summary of the major clinical manifestations of the ATR-X syndrome.

<table>
<thead>
<tr>
<th>Clinical feature</th>
<th>Total</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Profound mental retardation*</td>
<td>115/118</td>
<td>97</td>
</tr>
<tr>
<td>Normal birth weight</td>
<td>68/77</td>
<td>88</td>
</tr>
<tr>
<td>Neonatal hypotonia</td>
<td>65/75</td>
<td>87</td>
</tr>
<tr>
<td>Seizures</td>
<td>39/109</td>
<td>33</td>
</tr>
<tr>
<td>Characteristic face</td>
<td>92/103</td>
<td>89</td>
</tr>
<tr>
<td>Microcephaly</td>
<td>70/97</td>
<td>72</td>
</tr>
<tr>
<td>Genital abnormalities</td>
<td>88/103</td>
<td>85</td>
</tr>
<tr>
<td>Skeletal abnormalities</td>
<td>92/100</td>
<td>92</td>
</tr>
<tr>
<td>Cardiac defects</td>
<td>20/107</td>
<td>19</td>
</tr>
<tr>
<td>Renal/urinary abnormalities</td>
<td>18/106</td>
<td>17</td>
</tr>
<tr>
<td>Gut dysmotility</td>
<td>70/94</td>
<td>74</td>
</tr>
<tr>
<td>Short stature</td>
<td>54/83</td>
<td>65</td>
</tr>
<tr>
<td>HbH inclusions</td>
<td>88/100</td>
<td>88</td>
</tr>
</tbody>
</table>

119 patients: total represents no. of patients about whom appropriate information is available and includes patients who do not have thalassaemia but in whom ATRX mutations have been identified. *Patient too young (<1 year) to assess degree of mental retardation.
individuals. It seemed likely that the defect in globin synthesis lay in trans to the \( ^\text{a} \)-globin cluster. This was confirmed in a family with four affected sibs in whom the condition segregated independently of the \( ^\text{a} \)-globin cluster.\textsuperscript{9}

Preliminary observations indicated that the syndrome mapped to the X-chromosome and hence it was named the ATR-X syndrome. Subsequent linkage analysis of 16 families localised the disease to the region Xq13.1-q21.1 confirming that the associated \( ^\text{a} \)-thalassaemia results from a trans-acting mutation.\textsuperscript{10}

The ATR-X syndrome behaves as an X-linked recessive disorder; only boys are affected. Female carriers have a normal appearance and intellect, although approximately one in four carriers have subtle signs of \( ^\text{a} \)-thalassaemia with very rare cells containing HbH inclusions.\textsuperscript{10}

Identification of the ATR-X disease gene

The isolation of cDNA fragments mapping within Xq13.1-q21.1 provided the opportunity to study candidate genes for ATR-X.\textsuperscript{11} cDNAs (from M. Fontes et al.) isolated by direct selection using YACs mapping to this region were screened by hybridisation to DNA samples from a panel of patients. Using an 84 bp cDNA fragment, the hybridisation signal was found to be absent in one individual. Further mapping with genomic probes isolated with the cDNA probe indicated that this patient had a 2.0 kb deletion of which approximately 1.6 kb was subsequently shown to encode mRNA. The cDNA fragment was used to isolate further cDNA clones\textsuperscript{6} and eventually a contig spanning a substantial portion of the cDNA was isolated.

Characterisation of the ATRX gene and its potential role in gene expression

We now know that the ATRX gene spans about 300 kb of genomic DNA and contains 36 exons.\textsuperscript{12} It encodes at least two alternatively spliced \( \approx 10.5 \) kb mRNA transcripts which differ at their 5' ends and are predicted to give rise to slightly different proteins of 265 and 280 kD respectively (Figure 3).

Figure 2. (a) Haemoglobin levels; and (b) Mean cell haemoglobins in subjects with ATR-X syndrome at various ages. A solid line indicates the mean and a dashed line 2 SD below the mean (30). For any subject only one result within each consecutive 5-year period is given. (c) Proportion of red cells with HbH inclusions in subjects with ATR-X syndrome after 1% brilliant cresyl blue incubation at room temperature. Only one result is given for each subject. The percentage of positive cells is plotted on a logarithmic scale.
represents a major site of mutations in patients with ATR-X syndrome, containing over 60% of all mutations (Figure 3). Also within the N-terminal region is a segment of protein that has a high probability of forming a coiled coil, a structure implicated in protein-protein interactions. Although this resides in an evolutionarily less conserved region a similar coiled coil is predicted in the mouse protein. Using the yeast 2-hybrid approach Le Douarin and colleagues suggested that this region interacts with the mouse chromatin associated protein mHP1. The central and C-terminal regions show the greatest conservation between mouse and human sequences (94%). The central portion of the molecule contains motifs that identify ATRX as a novel member of the SNF2 subgroup of a superfamily of proteins with similar ATPase and helicase motifs. Other members of this subfamily are involved in a wide variety of cellular functions including the regulation of transcription (SNF2, MOT1 and brahma), control of the cell cycle (NPS1), DNA repair (RAD16, RAD54 and ERCC6) and mitotic chromosome segregation (Iodestar). An interaction with chromatin has been shown for SNF2 and brahma and may be a common theme for all this group. It is of considerable interest that ATRX is most similar to the protein M-i-2 which also shows similarity to ATRX in the PHD domain (see above). The consistent association of ATRX with thalassaemia suggests that the protein normally exerts its effect at one or more of the many stages involved in gene expression.

Different effects of ATR-X mutations on α and β globin expression

It is interesting that ATRX mutations appear to down-regulate expression of the α rather than the closely related β-globin genes. Activation of the α- and β-globin genes involves a common group of lineage-restricted (GATA-1 and NF-E2) and ubiquitous (CACC box) DNA-binding factors. A key difference, however, is that the α-globin genes lie in a region of constitutively open, transcriptionally active chromatin and their expression is regulated by a remote tissue-specific enhancer, whereas the β-globin genes lie within a segment of chromatin that is closed in non-erythroid cells but which opens in a tissue-specific manner under the influence of a remote locus control region. It is conceivable that the ATRX protein

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Figure 3. Schematic diagram of the complete ATRX cDNA (upper figure), the boxes represent the 36 exons, thin horizontal lines represent the introns (not to scale). The largest ORF is shown as an open box (lower figure) with the 5' and 3' UTR sequences as black lines with the poly(A) tail denoted (An). The positions of the mutations are shown by circles; open circles indicate mutations that would cause protein truncation. The alternate splicing that results in transcripts lacking exons 6 and 7 or lacking exon 7 only is shown by inverted Vs. The alternate initiation codons are labelled M1 and M2. The principal domains, the N-terminal hydrophilic domain, the central domain containing the highly conserved helicase motifs and the C-terminal domain are indicated. Additional domains are shown as black boxes and include the zinc finger motif (ZFM), the potential coiled coil (CC), a stretch of 21 glutamic acid residues (E), the P box (P) and a glutamine rich region (Q). The lower part of the figure shows a graphical representation of the amino acid similarity between human and mouse ATRX proteins.
acts to derepress or activate the \( \alpha \)-globin genes by interaction with chromatin whereas these functions aresubserviced by alternative mechanisms in the \( \beta \)-globin cluster. The analysis of \( \alpha \)-globin regulation and its perturbation by ATRX mutations offers aunique opportunity to understand exactly how the protein regulates gene expression.

In addition to \( \alpha \)-thalassemia, patients with the ATR-X syndrome have multiple associated congenital abnormalities, it seems likely that ATRX mutations exert pleiotropic effects. By analogy with SNF2, for example, ATRX may regulate expression of a restricted class of genes; perturbation in expression of these target genes could affect development of many different systems, including the central nervous system.

Making a connection with chromatin

Several lines of evidence, set out above, suggest that ATRX could normally affect gene regulation via an effect on chromatin. To examine further the relationship between ATRX and chromatin in vivo we have developed a panel of antibodies to determine its distribution in the nucleus during interphase and mitosis. Several lines of evidence show that a substantial proportion of ATRX associates with pericentromeric heterochromatin and in human chromosomes at metaphase there is a striking and unexpected association between ATRX and the short arms of acrocentric chromosomes where the GC-rich ribosomal DNA (rDNA) arrays are found.20

Pericentromeric localisation has now been reported for a number of proteins which play multiple roles in modifying chromosomal structure and function. In Saccharomyces cerevisiae the centromere promoter factor (called CP1 or CPF1) may modulate chromatin structure at many promoters, where it is necessary for optimal gene expression, but it is also found at centromeres where it is thought to facilitate the binding of kinetochore proteins.21 CP1 mutants show abnormalities in centromere function and gene expression.22 In Drosophila, GAGA factor, a product of the Trithorax-like locus, binds hundreds of euchromatic loci, where in association with the nucleosomal remodelling factor (NURF), it facilitates transcriptional activation.23 GAGA factor is also associated with pericentromeric heterochromatin in diploid cells where it plays a role in chromosome condensation and segregation.24 In mammals, it has recently been shown that Ikaros, a transcriptional regulator required for normal lymphocyte development, may act as repressor when localised with M-i-2 and HDAC to heterochromatin,25-26 and as an activator with SWI/SNF in euchromatic regions.25 Similarly, ATRX may have multiple (positive and negative) effects on chromatin mediated processes and this may provide the basis for the presence of a thalassaemia and disturbed patterns of expression for many other genes. At present we do not know whether ATRX plays a direct functional role at heterochromatin and rDNA repeats. However, by binding ATRX, the repeats could provide buffers which regulate the amount of the protein available to its biologically important sites. This may be analogous to previous observations in Drosophila in which deletion or amplification of rDNA or satellite DNA repeats enhance or suppress position effect variegation by varying the amount of the modifier proteins that are bound.27,28 Polymorphic variation in the numbers of such repeats would provide a plausible explanation for the clinical variability seen in patients with identical mutations of the ATRX gene producing a structurally normal but reduced amount of ATRX.12,29

Conclusions

Understanding the ATR-X syndrome has opened up many new questions. It seems likely that mutations in this class of chromatin-associated genes which appear to regulate many other genes may be a common cause of syndromal mental retardation and we hope that over the next few years using information gathered from the ATR-X syndrome we will identify other similar conditions. At the same time it will be important to identify the ‘target’ genes other than \( \alpha \)-globin that are regulated by ATRX. Finally, having established that ATRX is almost certainly involved in the normal regulation of \( \alpha \) gene expression we want to understand exactly what role it plays in this process.

References


Pathophysiology of paroxysmal nocturnal haemoglobinuria

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The phrase paroxysmal nocturnal haemoglobinuria (PNH) describes the most dramatic manifestation of a blood disorder which we must now regard as quite complex. Indeed, haemoglobinuria results from haemoglobinuria which results from intravascular haemolysis; therefore PNH has been correctly classified as a haemolytic anaemia. However, the anaemia is often also associated with a decrease in neutrophils, or platelets, or both, hinting at a broader pathology of the haematopoietic system. In this paper I wish to review the evidence that PNH results from a combination of qualitative and quantitative changes in the bone marrow.

Epidemiology

PNH is encountered in all populations throughout the world, and it can affect people of all ages and all socio-economic groups; however, it has never been reported as a congenital disease, and to the best of our knowledge there is no report of family clustering. Thus, PNH is an acquired disease. There is little information on the population incidence of PNH: however, this is estimated to be 5-10 times less than that of aplastic anaemia (AA): thus, PNH is a rare disease. It has been suggested that, like AA, PNH may be somewhat less rare in South East Asia and in the Far East.1

Clinical features

In most cases2 the patient presents as a problem in the differential diagnosis of anaemia, whether symptomatic or discovered incidentally. The diagnosis is immediately made much easier if the patient reports having passed dark urine, due to haemoglobinuria, the landmark of intravascular haemolysis. In some patients PNH may present with typical signs and symptoms of thrombosis in a deep vein in one of the limbs; in others with recurrent attacks of severe abdominal pain, which may prove to be due to intra-abdominal thrombosis. The most classical site is in the hepatic veins, causing the Budd-Chiari syndrome; but the mesenteric vessels or the splenic or the portal vein may be also involved. Not infrequently, the anaemia is associated with other cytopenias: this suggests some degree of bone marrow failure (BMF). Indeed, sometimes a PNH patient may become less haemolytic and more pancytopenic, converting in fact to aplastic anaemia (AA). Conversely, PNH can emerge in patients with a previous diagnosis of AA: indeed, in some recent series small PNH clones have been detected in up to 50% of cases of bona fide AA.3

The natural history of PNH is that of a very chronic disorder, which may affect the patient continuously for decades. Without treatment the median survival is estimated to be about 8 years; in the past the commonest causes of death have been thrombosis or haemorrhage associated with severe thrombocytopenia. Rarely, PNH may terminate in acute myeloid leukaemia. On the other hand, full spontaneous recovery from PNH has been also well documented.4 Thus, in discussing the pathophysiology of PNH in relation to its clinical manifestations, we have three main topics to consider: (i) haemolysis, (ii) thrombosis and (iii) BMF.

Haemolysis

Haemolysis in PNH is due to an intrinsic abnormality of the red cell. This was first characterised serologically, through the finding that, unlike in auto-immune or allo-immune haemolytic anaemia, there was no specific antibody involved. Rather, the red cells undergo haemolysis whenever they are in the presence of activated complement (C), whether it is activated by an antibody (e.g. anti-I, present at low titre in most normal people), or through the alternative pathway (e.g., by lowering pH or ionic strength). Indeed, acidification of serum was used to develop a diagnostic test that is still valid;5,6 and low ionic strength has been used for a screening test (sucrose haemolysis) which should be probably now regarded as obsolete. It took half a century before the biochemical basis for this peculiar hypersusceptibility to C was clarified. We now know that several membrane proteins protect cells – including red cells – against damage from the membrane attack complex of C (C5-C9). One of these proteins, CD59, specifically hinders the insertion into the membrane of C9 polymers.7,8 CD59 is severely deficient or completely absent from the membrane of PNH red cells.9 This explains why there is chronic haemolysis in PNH, why the haemolysis is largely intravascular, and why it can be dramatically exacerbated in the course of a viral or bacterial infection, when antigen-antibody reactions associated with the infection will cause bursts of C activation.
Biochemical abnormalities and somatic cell mosaicism
Serological studies had suggested long ago that certain normal antigens were poorly expressed on the surface of red cells. With the introduction and increasing use of flow cytometry, it was discovered that a bewildering multitude of membrane proteins is deficient in the abnormal population of blood cells of different lineages in patients with PNH. In most cases there is no obvious similarity in the function of these proteins; rather, they have a common structural element, namely a phospholipid moiety; specifically, a glycosyl-phosphatidylinositol (GPI) which includes an ethanolamine which can form a peptide link with a C-terminal amino acid of certain proteins. Because this lipid is embedded in the lipid bilayer of the membrane, and serves to retain the protein attached to the membrane, it is referred to as a GPI anchor. The fact that all GPI-linked proteins are deficient on the membrane of PNH cells suggested that the underlying defect might be in the complex biochemical pathway through which the GPI is synthesized in mammalian cells.

A remarkable feature of patients to PNH, when compared with those who have haemolytic anaemia due to some other intracutaneous cause, is that not all their red cells participate in the haemolytic process: indeed, some of their red cells are qualitatively normal. This led to the notion that PNH is a clonal disorder due to a somatic mutation. Direct experimental evidence supporting this notion was obtained three decades ago in PNH patients who were heterozygous for the X-linked gene encoding glucose 6-phosphate dehydrogenase, and in whom all PNH cells expressed the same G6PD allele. Thus, we have true somatic cell mosaicism in patients with PNH, and this has not made it easy to identify the biochemical abnormality of PNH, since patient material always consists of a mixture of both types of cells. However, a viable approach was to study cloned lymphoblastoid cell lines (LLCL) that displayed the PNH phenotype, obtained from PNH patients, and to use as controls LLCL with normal phenotype obtained from the same patients. Comparative analysis of these two sets of cell lines revealed normal synthesis of phosphatidylinositol, but failure to incorporate mannose or NAcGlcN, thus pinpointing a block at the level of the NAcGlcN transfer.

Molecular genetics
The PIG-A gene (so called for the phosphatidyl inositol glycan complementation group A) was isolated by expression cloning, i.e. through its ability to restore the expression of GPI-anchored proteins on the surface of cells lacking those proteins, including those from PNH patients. PIG-A maps to the short arm of the X chromosome on Xp22.1, within a region almost completely covered by physical contigs. It became quickly clear that acquired mutations of the PIG-A gene are responsible for PNH; to date, a total of 174 somatic mutations in the PIG-A gene have been identified by different investigators in more than 28 reports on 146 patients. Of these, (a) 135 are such (large deletions, frameshifts, nonsense mutations) that we can predict complete functional inactivation of the PIG-A gene product (PIG-Ao); (b) 35 are missense mutations and 4 are small in frame deletions. These two groups presumably account for the existence in PNH patients of blood cells with complete deficiency of GPI-anchored proteins (PNH III) or partial deficiency of GPI-anchored proteins (PNH II). The two types not infrequently co-exist in the same patient, indicating that two different clones are present; PNH III red cells are naturally even more susceptible to C than PNH II red cells, and therefore the absolute and relative amounts of these and of the residual normal red cell population affect the rate of haemolysis considerably.

The predicted protein product of PIG-A consists of 484 amino acids. A hydrophobic region near the carboxyl terminus may be a transmembrane domain (amino acids 415-442). The hydrophobic carboxyl terminal region of 42 residues corresponds to the luminal domain of PIG-A. Watanabe et al. have shown that PIG-A, together with 3 other proteins (PIG-H, PIG-C and GPI1) constitutes a complex that mediates the first reaction step of the GPI anchor biosynthesis, i.e. the transfer of GlcNAc onto phosphatidylinositol: this is in keeping with the biochemical studies mentioned above. The fact that PIG-A is part of an enzyme is also in keeping with the fact that PNH mutations are recessive.

Thrombosis
Thrombosis is one of the most immediately life-threatening complications of PNH, and yet one whose pathogenesis is least understood. In principle, we can envisage three sorts of mechanisms: a) impaired fibrinolytic. The urokinase plasminogen activator receptor (uPAR) is a GPI-linked protein, and it is deficient in leukocytes belonging to the PNH clone. If leukocytes play a role in endogenous fibrinolysis, PNH leukocytes might be less efficient in this function due to their inability to bind urokinase. In addition, the levels of soluble uPAR (which may normally help to regulate urokinase activity) are significantly increased in PNH patients. This could result in reduced endogenous fibrinolysis. However, we must remember that tissue plasminogen activator is believed to be the main effector of the conversion of plasminogen to plasmin; b) hyper-coagulability. The coagulation cascade, and therefore ultimately thrombin generation may become activated in PNH. For instance, the C5b-9 complex has been shown to induce release of platelet micro-particles that express receptors for factor Va and exhibit prothrombinase and “tenase” activity. It has been also held that intravascular haemolysis may release from red cells substances that have thromboplastin activity. However, in a series of 11 PNH patients, no abnormalities were seen in levels of thrombin/anti-thrombin complexes or in thrombin activation fragment F1.2.13 We must also admit that thrombosis can occur in PNH patients even when they are fully anti-coagulated with warfarin or hirudin, both of which would be expected to effectively prevent hypercoagulability through their effects on the common coagulation pathway. Therefore, activated coagulation pe se is not likely to be the main culprit for thrombosis in PNH; c) hyper-activity of

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platelets. After treatment with C5b-9 PNH platelets undergo increased vesiculation and thrombin generation, and elevated levels of platelet derived microparticles have been demonstrated in some PNH patients. The expression of activation markers on the surface of platelets from PNH patients is increased, probably because of abnormal C regulation, which may result in platelet activation. The lack of CD59 on the PNH platelet could lead to abnormal insertion of the C5b-9 complex in the platelet membrane, as is the case with the red cell.

Though all of these 3 factors may play roles in producing a thrombophilic state in PNH, it seems very likely that the primary cause lies in the PNH platelets, which are abnormal precisely because they belong to the PNH clone. This notion is supported by the occurrence of cerebral infarction in a patient who did not have PNH, but who had congenital CD59 deficiency and chronic hemolysis.

Bone marrow failure

The close association between PNH and AA (mentioned above) may reflect a shared pathogenetic basis. AA is essentially an organ-specific autoimmune disease. In the peripheral blood and in the bone marrow of patients with AA it is possible to find increased numbers of ‘activated’ CD8+ T-lymphocytes, which are able to inhibit growth in vitro of both autologous and HLA-identical haematopoietic colonies. T-lymphocytes might be implicated in causing AA in two ways. On the one hand, by secreting interferon-γ and tumour necrosis factor-β, they cause induction of Fas expression on CD34+ cells. On the other hand, cytotoxic T-lymphocytes (CTLs) may ultimately cause apoptosis through Fas-FasL interaction (although this sequence of events has not yet been conclusively demonstrated), leading to depletion of HSC. The putative auto-antigen that incites and serves as a target for the auto-reactive CTLs is not known. Perhaps the strongest evidence for the immune basis of AA is that the majority of patients respond to immuno-suppressive treatment.

Although PNH has elements of BMF, it is obviously different from AA because of its other prominent clinical features (haemolysis and thrombosis). One possible way to look at the pathophysiology of PNH is that it results precisely from the co-existence of BMF with a large PIG-A mutant clone. In order to explain the co-existence of these two components we can surmise, in principle, three possibilities: i) the

![Diagram of Diagnosis of PNH]

Figure 1. Guidelines for the management of PNH. This algorithm is based on the consideration that patients with this condition vary considerably (a) in terms of clinical severity, and (b) in terms of the contributions of the PNH clone and BMF respectively to determining the overall clinical picture. Some patients have been cured by bone marrow transplantation (BMT): at the other hand of the spectrum, some of the patients who for a long time have been living with PNH - by choice or otherwise - have eventually experienced spontaneous recovery.
PNH clone and BMF coexist by sheer coincidence; ii) the PNH clone causes BMF; iii) BMF favours the development or expansion of the PNH clone. Because AA and PNH are both rare diseases, the first possibility can be discarded on statistical grounds as being too improbable. The second possibility is unlikely, since PNH often develops in patients originally suffering from AA, who therefore already had established BMF at a time when no PNH clone could be demonstrated. Thus, the third possibility seems the most likely, and it has recently obtained support from experimental findings in mice, as well as observations in humans.

Consequences of PIG-A inactivation in mice
When the PIG-A gene is targeted by homologous recombination in mouse embryonic stem (ES) cells, and these are then injected into blastocysts, viable chimeric animals can be obtained only if the contribution to the embryo from the PIG-A null cells is low, and the numbers of GPI-negative cells in their blood is also low. Recently by using the technique of conditional ‘knock-out’, based on what is known in jargon as the cre-lox system, two groups have succeeded in producing mice that can be regarded as true models for human PNH. Indeed, the mice have two discrete populations of red cells, granulocytes, monocytes, and lymphocytes: in first approximation, the flow cytometry patterns are remarkably similar to those seen in patients with PNH. In addition, although the mice are not anaemic, they do have evidence of haemolysis and their red cells have increased susceptibility to complement (data on platelets and thrombosis are not yet available). Interestingly, the clinical course of the mice is very benign, and the proportion of PNH cells is remarkably stable, in spite of

Figure 2. The role of somatic mutation and auto-immune mediated bone marrow failure in the pathophysiology of PNH. The top panel is a cartoon of normal hematopoietic stem cells (HSC): the arrow indicates a somatic mutation in the PIG-A gene in one of the HSC. As a result, the cell and its progeny lose surface GPI-anchored proteins. As time goes on, micro clones arising from such mutant cells may become exhausted, and new ones may arise: however, there is no clonal expansion. The middle panel illustrates the presence in the bone marrow of auto reactive immune cells, which may be cytotoxic T cells: these attack the HSC, which gradually decrease in numbers, eventually resulting in the picture of aplastic anemia (AA). The bottom panel illustrates the consequences of the co-existence of a PIG-A somatic mutation and auto reactive immune cells in the bone marrow. If we make the specific hypothesis that the target of the auto immune attack is a GPI-anchored protein, the mutant clone will expand as a result of negative selection against the normal HSC. As a result, the majority of hematopoiesis will consist of GPI deficient cells. The large numbers of C-susceptible red cells will cause hemolytic anaemia; the large numbers of abnormal platelets will cause a high risk of thrombotic complications.
the fact that these mice are born with a large number of pre-formed PNH cells. In humans, by contrast, before giving the clinical picture of PNH, the PNH cell population must have undergone substantial expansion, since it arises from a single mutant stem cell.\(^{39}\) It is tempting to surmise that the PNH cells in mice are very similar to PNH cells in human patients, but that the mice do not have the human disease, PNH, because they do not have BMF. The fact that BMF is lacking supports the notion that its causation is independent of the PNH cell population itself.

PNH clones and micro clones in humans

We have already mentioned that PNH III and PNH II can co-exist in the same patient; indeed, by mutation analysis it is not infrequent to find two or more clones in the same patient. These findings are strongly suggestive of the notion that these clones expand, simultaneously or in sequence, in response to a certain selective agent present in the patient’s bone marrow environment.

On the other hand, one can ask the complementary question: Is there evidence for PNH clones in normal people, and what is the fate of such clones? It has been shown recently that very small populations of PNH granulocytes and PNH erythrocytes are present in normal people, and these are probably generated simply by the ‘background’ level of somatic mutations in the PIG-A gene. Exactly as in patients with PNH, missense, frameshifts, and nonsense mutation have been identified. Two of these mutations had been found previously in patients with classical PNH, thus showing formally and conclusively that PIG-A gene mutations are not sufficient for the development of PNH.\(^ {36}\) The fact that these very tiny PNH cell populations— which we could call micro clones — do not expand clearly means that they do not have any growth advantage on their own. At the same time, this fact suggests that in PNH patients the same sort of clones have expanded because they found an environment favourable to themselves.

Some clinical implications

Classification of PNH

How well do these concepts fit the clinical reality of patients with PNH? This can be tested by considering, in each patient, the relative roles of the PNH clone(s) and of BMF in determining the clinical picture. At one end of the spectrum we may find a patient in whom the PNH clone is so large that it masks BMF (almost) completely. The patient will have signs and symptoms of brisk haemolysis and may have serious thrombotic complications, but the peripheral blood count is normal or near normal; the patient can be said to have florid PNH. At the other end of the spectrum we may find a patient who meets all criteria for severe AA, and who is found (by sensitive analytical techniques) to have in addition a very small proportion of GPI-negative cells in the blood. In such a patient the presence of the PNH clone is not likely to affect the clinical course significantly, and therefore we prefer to designate the patient as having AA with a PNH clone, because it is the BMF rather than the PNH that must dictate therapeutic decisions. Intermediate situations are not uncommon. Most importantly, since there is a dynamic relationship between PNH clone(s) and BMF, transitions from one form to another may take place.

Therapy

It is clear from the above that there are two problems to deal with in a patient with PNH: the PNH clone and BMF: inevitably this must affect the approach to treatment (Figure 1). Indeed, in first approximation the management of patients with what we have called AA with a PNH clone does not differ significantly from that of patients with straightforward AA. In contrast, patients with florid PNH may present unique clinical problems, mainly massive haemolytic attacks and serious thrombotic complications. For these patients the two extreme choices are radical treatment by bone marrow transplantation (BMT) or supportive treatment only (in which red cell transfusions must often be a major component). When an HLA-identical sibling is available, allogeneic BMT is probably the treatment of choice for younger patients with moderate to severe cytopenias, and in view of the risk of life-threatening complications it must be regarded as an option to be offered to any young patient with PNH. In contrast, the past record of BMT from unrelated donors in PNH is poor, and it must still be regarded as experimental in the treatment of this condition. For patients who do not have an appropriate donor (and perhaps also for those who do) immuno-suppressive treatment with ALG (ATG) and cyclosporin A may be a good alternative. This type of treatment cannot be expected to eradicate the PNH clone, because it is aimed at relieving the immune-mediated inhibition of normal haematopoiesis: however, in doing this it will limit, if not eliminate, the abnormal marrow environment in which the PNH clone thrives. Thus, in treating the BMF component of PNH we can capitalise on what has been learnt from AA; for treating the consequences of having a large PNH clone (haemolysis and thrombosis) we urgently need to develop more imaginative approaches than are currently available.

Conclusion: a coherent model for the pathogenesis of PNH

In terms of the nosographic classification of human diseases PNH is rather special and indeed perhaps unique for at least two reasons: a) although it results from somatic mutations, it is not per se a malignant disease; b) it is probably the only form of acquired haemolytic anaemia which is due to an intrinsic red cell abnormality.

On the basis of current knowledge, we can formulate the following model for the pathogenesis of PNH (Figure 2) which explains most of its clinical and haematological features: 1) PNH always co-exists with BMF; 2) BMF is clinically obvious in patients who initially present with AA and then develop PNH. In patients who initially present with PNH, BMF may not be obvious because by the time of diagnosis the PNH clone has expanded to the point at which it provides a substantial proportion of the patient’s haematopoiesis; 3) The PNH clone has a long but probably finite life span. If, by the time the PNH clone is exhausted, BMF has not recovered, the patient
evolves clinically from PNH to AA. If, by the time the PNH clone is exhausted, BM F has recovered, the patient will be "cured" of PNH; 4) A PNH clone arises through a PIG-A mutation in a HSC. Since there is only one active X-chromosome in each HSC, the mutated stem cell and its progeny will acquire the PNH phenotype, and this can happen in any normal person. Cells with the PNH phenotype have no intrinsic growth advantage, and therefore PNH clones will not normally expand. As long as there is no BMF, clinical PNH will not develop; 5) The existence of a florid PNH clone while the rest of hematopoiesis is depressed suggests that the PNH clone can be spared selectively from the injury affecting the rest of the bone marrow; 6) in order to explain point (5), we may surmise that the damage to stem cells causing BMF is mediated through a GPI-linked surface molecule. In this case, the PNH cells lacking these molecules will survive; 7) thus, the very defect of the PNH clone may endow it with a relative survival or growth advantage in a patient with BMF. If the patient has such a clone, he or she will present with PNH; otherwise he or she presents with overt AA. Thus, the development of PNH is conditional on a background of BMF; 8) a large PNH clone carries with it intravascular haemolysis and thrombosis, the "classical" manifestations of PNH.

Acknowledgments

I thank all my colleagues with whom I have been fortunate to work on PNH over the years. I am very grateful to D. Aratlar haemolysis and thrombosis, the 'classical' manifestations of PNH.

References