Hyaluronan in normal and malignant bone marrow

A clinical and morphological study with emphasis on

myelofibrosis

GUNNEL SUNDSTRÖM

Umeå 2006
Cover illustration: Hyaluronan (HYA) in the bone marrow from a patient with idiopathic myelofibrosis. The HYA staining is brown. The pattern is diffuse and intense, score 4. Magnification x 40 (Sundström et al 2002).
Till min familj
Jag har inte sanningen,

jag söker den

K-G Hammar ärkebiskop
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ABSTRACT
Hyaluronan in normal and malignant bone marrow.
A clinical and morphological study with emphasis on myelofibrosis.
Gunnar Sundström, Department of Public Health and Clinical Medicine, Umeå University, S-901 85 Umeå, Sweden.

Fibrosis in the bone marrow is usually denominated myelofibrosis and may contribute to impaired hematopoiesis. Myelofibrosis is seen both in malignant and non-malignant diseases. The normal microenvironment in the bone marrow consists of a heterogenous population of hematopoietic and non-hematopoietic stromal cells, their extracellular products and hematopoietic cytokines. The stromal cells produce a complex array of molecules, among others collagens and glycosaminoglycans (GAGs) of which hyaluronan (HYA) is the most abundant. Marrow fibrosis results from an increased deposition of collagens, which are polypeptides. Staining for reticulin, mostly composed of collagen type III, is the common way of visualizing myelofibrosis. HYA, like the collagens, is widely distributed in connective tissues. Little is known about the distribution of HYA in bone marrow.

The aims of this thesis have been to determine how HYA is distributed in normal and malignant bone marrow, compared to reticulin staining, and to follow patients with chronic myeloproliferative diseases (CMPD) during two years treatment with anagrelide considering development of cellularity and fibrosis.

In bone marrow biopsies from healthy volunteers, the controls, HYA was found in a pattern that was concordant with the reticulin staining.

Comparing patients with different malignant diseases with and without bone marrow involvement, HYA staining was found to be significantly stronger in both groups compared to the controls.

The HYA scores were also significantly higher in the bone marrow of patients with de novo acute myeloid leukemia (AML), compared to the controls.

There was a correlation between HYA and reticulin in the patients with de novo AML, and in the patients with different malignant diseases with and without bone marrow involvement as in the controls.

Increase of HYA, reticulin and cellularity in the bone marrow of patients with CMPD after two years of treatment with anagrelide indicated progression of fibrosis. Anagrelide is a valuable drug for reduction of platelets but seems unable to stop progression of fibrosis and hypercellularity.

HYA is an interesting molecule with properties not only contributing to the structure of extracellular matrix but also to cell signaling and behaviour, although the understanding of the detailed mechanisms is still incomplete.

Keywords: hyaluronan, reticulin, fibrosis, chronic myeloproliferative disorders, acute myeloid leukemia, anagrelide, bone marrow

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ABBREVIATIONS

ALL  acute lymphoblastic leukaemia
alloSCT  allogeneic stem cell transplantation
AML  acute myeloid leukaemia
APL  acute promyelocytic leukaemia
ATRA  all-trans retinoic acid
autoSCT  autologous stem cell transplantation
CEL  chronic eosinophilic leukaemia
CIMF  chronic idiopathic myelofibrosis
CLL  chronic lymphocytic leukaemia
CML  chronic myeloid leukaemia
CMML  chronic myelomonocytic leukaemia
CMPD  chronic myeloproliferative disease
CMPD,U  chronic myeloproliferative disease, unclassifiable
CNL  chronic neutrophilic leukaemia
DLBCL  diffuse large B-cell lymphoma
ECM  extra cellular matrix
EEC  endogenous erythroid colony
EGF  epidermal growth-factor
ET  essential thrombocythemia
FAB  French-American-British
bFGF  basic fibroblast growth factor
GAG  glycosaminoglycan
GVHD  graft-versus-host-disease
GVL  graft-versus-leukaemia
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>HABP</td>
<td>hyaluronan binding protein</td>
</tr>
<tr>
<td>HCL</td>
<td>hairy cell leukaemia</td>
</tr>
<tr>
<td>HYA</td>
<td>hyaluronan</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase gene</td>
</tr>
<tr>
<td>MDS</td>
<td>myelodysplastic syndrome</td>
</tr>
<tr>
<td>MMM</td>
<td>myeloid metaplasia with myelofibrosis</td>
</tr>
<tr>
<td>MRC-PT1</td>
<td>Medical Research Council Primary Thrombocythaemia 1</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>PRV</td>
<td>polycythemia vera rubra</td>
</tr>
<tr>
<td>PV</td>
<td>polycythemia vera</td>
</tr>
<tr>
<td>PVSG</td>
<td>Polycythemia Vera Study Group</td>
</tr>
<tr>
<td>REAL</td>
<td>Revised European-American Lymphoma</td>
</tr>
<tr>
<td>RES</td>
<td>reticuloendothelial system</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
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</table>
ORIGINAl PAPERS

This thesis is based on the following publications and manuscripts, which will be referred to in the text by their Roman numerals:


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INTRODUCTION

Fibrosis in the bone marrow is usually denominated myelofibrosis, but this term is also used for a specific myeloproliferative disorder, chronic idiopathic myelofibrosis (CIMF). In this presentation, the term myelofibrosis is used to denominate the bone marrow process, irrespectively of underlying disorder, and referring to an increased amount of connective tissue in the bone marrow. Fibrosis in the bone marrow may contribute to impaired hematopoiesis and is associated with both malignant and non-malignant diseases (Reilly 1997).

Studies during the last decades have increased the understanding of the complexity of both normal and myelofibrotic stroma in the bone marrow. Marrow fibrosis results from an increased deposition of different collagens, which are polypeptides forming a triple helix structure, and neovascularization. Glycosaminoglycans (GAGs) of which hyaluronan (HYA) is the most abundant and different glycoproteins like fibronectin, laminin, tenascin and vitronectin also contribute to development of fibrosis (Fig 1). It is known that megakaryocyte/platelet-derived growth factors play an important role in enhancing the fibroblast proliferation and ultimately the accumulation of collagens. Platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF), mainly produced by megakaryocytes, have been implicated in the proliferation of fibroblasts and the pathogenesis of bone marrow fibrosis. Even monocytes and/or macrophages, which are major sources of cytokines, may be involved in development of fibrosis (Apaja-Sarkkinen et al 1986, Lisse et al 1991, Hasselbalch 1993, Reilly et al 1995, Reilly 1997, Schmitz et al 1998, Rameshwar et al 1994, Sadahira et al 1999).
HYA is a large hydrophilic molecule and provides stability and elasticity to the extra-cellular matrix (ECM) and its role in fibrosis has been discussed. It has been demonstrated that PDGF, EGF, TGF-β and bFGF stimulate HYA synthesis (Engström-Laurent et al 1985a, Heldin et al 1989). It has also been shown that circulating HYA and collagen type III in serum correlate significantly. Type III collagen can be demonstrated in serum by the application of antibodies against type III procollagen (Hasselbalch et al 1991, Reilly et al 1995).

Scoring systems for grading of myelofibrosis are mainly based on subjective evaluations by individual pathologists. The most frequently used grading system of reticulin staining is based on the Bauermeister six-grade scale (Bauermeister 1971). Experienced hematopathologists recently published guidelines for measuring bone marrow cellularity and fiber content on a four-grade scale (Thiele et al 2005). The intensity of histochemical staining of HYA is evaluated on a four-grade scale (Sundström et al 2002). Bone marrow core biopsy is required for demonstrating myelofibrosis and provides the best way to show the architecture of the bone marrow.

In normal bone marrow the ECM is mainly composed of collagens (type I, III, IV, V), proteoglycans and different GAGs. Collagen type I forms coarse fibres and is most abundant.
in the vessel walls but also occurs in the bone marrow. Collagen type III forms thin fibres and is scattered throughout the bone marrow and around blood vessels.

Microscopic examination of core biopsies that have been stained for interstitial reticulin is the common way of visualizing fibrosis in the bone marrow. Reticulin has been shown to be composed mostly of type III collagen. It has been documented that HYA is distributed along the reticulin net (Smadja–Joffe et al 1996, Sundström et al 2002). Collagen type IV is visualized discontinuously in the basement membrane lining the sinusoids. Laminin and type IV collagen are closely co-distributed in normal bone marrow (Lisse et al 1991).

Myelofibrosis is common in patients with chronic myeloproliferative diseases (CMPDs). In the subgroup of patients with CIMF, also called myeloid metaplasia with myelofibrosis (MMM), myeloproliferation and myelofibrosis are characteristics of the disease. The myeloproliferation results from a clonal amplification of progenitor cells and the cytokines PDGF, TGF-β, and bFGF, produced by stromal cells, morphologically abnormal megakaryocytes and platelets, are proposed to be involved in the abnormal proliferation of polyclonal fibroblasts. The process results in myelofibrosis with deposition of collagens, HYA, fibronectin, vitronectin, laminin and tenascin (Thiele et al 1991, Le Bousse-Kerdiles et al 1999, Schmitz et al 1998). HYA and type III collagen metabolites in serum have been measured and found to be increased in CMPD patients with active disease (Hasselbach et al 1991). In bone marrow with extensive fibrosis the abnormal megakaryocytes usually remain even when the other blood cells have disappeared. Even in some lymphoproliferative diseases, e.g. hairy cell leukemia, an increased amount of fibrosis is regularly seen in the bone marrow (Burke et al 1974, Estey et al 1992, Juliussen et al 1992). Myelofibrosis is occasionally seen in acute myeloid leukaemia (AML), especially in acute megakaryoblastic
leukaemia, but there is scarce information about the frequency of myelofibrosis in the other subtypes of leukaemia. Whether fibrosis in AML is a poor prognostic factor and of importance for outcome has been discussed (Manoharan et al 1979, Islam et al 1984, Fohlmeister et al 1988, Thiele et al 1991, Islam 1993).

The myelodysplastic syndromes (MDSs) are disorders with heterogenous clinical courses. Myelofibrosis occurs in all subgroups of MDS, however with a higher incidence in chronic myelomonocytic leukemia (CMML). Myelofibrosis in MDS seems to herald a poor prognosis with reduced life expectancy and a high rate of transformation to acute leukemia (Maschek et al 1992, Marisavljevic et al 2004).

Myelofibrosis also occurs in association with non-malignant conditions, e.g. infections. Several cases of tuberculosis with myelofibrosis have been reported (Donhauser 1908, Samuelsson et al 1966).

There is no specific treatment to stop or reverse myelofibrosis. However, the process is reversible, contrary to what was generally believed until rather recently. The process stops if the cell(s) that are responsible for the production of cytokines which trigger the fibrosis are eliminated. It has been demonstrated that normalization of the bone marrow architecture and function can be achieved if the underlying disorder is eradicated. This was first observed in some patients with myelofibrosis associated with acute leukaemia that entered complete remission following chemotherapy, and reversal of myelofibrosis is now regularly observed in patients with MMM who are successfully treated with allogeneic stem cell transplants (Kelemen et al 1977, McGlave et al 1982, Smith et al 2001, Jurado et al 2001).
Like the collagens, HYA is widely distributed in the extra cellular matrix. It is possible to localise HYA histochemically in tissues, which has been done in different organs, mostly in animals but also in humans. However little is known about the distribution in bone marrow. The aim of this thesis has been to determine the distribution of HYA in normal and malignant bone marrow, compared to reticulin staining which takes into account only the end-result, established fibrosis. Our hypothesis is that synthesis and deposition of HYA is an early event in the formation of myelofibrosis and that quantification of HYA in combination with reticulin may be a tool to study the dynamics and velocity of the process.

BACKGROUND

Bone marrow

The bone marrow is one of the largest organs in the human body and the principal site of blood cell formation. In the normal adult its daily production is about 2.5 billion red cells, 2.5 billion platelets, and 1.0 billion granulocytes per kilogram of body weight in addition to lymphocytes. The rate of production is adjusted to actual needs and varies from nearly zero to many times normal (Testa et al 1993). At birth all bones contain hematopoietic marrow. Fat cells begin to replace hematopoietic marrow in the extremities in the fifth to the seventh year, and by adulthood the hemopoietic marrow is limited to the axial skeleton and the proximal portion of the extremities. The marrow fills the spaces between the trabeculae of bone in the marrow cavity. It is soft and friable and can readily be aspirated or biopsied with a needle. Marrow biopsy is particularly valuable for estimating marrow cellularity and detecting myelofibrosis and infiltrative diseases as lymphoma and carcinomatous invasion. Most marrow aspirates and biopsies are obtained from the iliac crest at the posterior superior spine. In adults even the sternum can be used for aspirates (Ellis LD et al 1964, Jensen WN et al 1964, Jamshidi et al 1971, Bearden JD et al 1974, Pasquale D et al 1986). Repeated biopsies
are useful to follow the course of disorders that are commonly associated with fibrosis, e.g. the CMPDs (Winfield et al 1992).

Studies of marrow cells have shown that cell lines consist of mature end cells with a finite functional life-span, capable of limited proliferation before their full maturation but without the capacity for self-renewal. The pluripotent hematopoietic stem cells give a continuous production of blood cells: erythroblasts, myeloblasts, megakaryoblasts, monoblasts, and lymphocytes (Fig 2). Sustained cellular production depends on the presence of stem cells with the capacity of continuous self-renewal. Stem cells circulate in the blood and can reenter the marrow and reestablish hemopoiesis in the marrow cords. Marrow cells from a histocompatible allogeneic donor can re-enter the marrow and reconstitute hemopoiesis of a recipient (Maloney et al 1972).
Bone marrow microenvironment

The normal microenvironment in the bone marrow consists of a heterogeneous population of hematopoietic and nonhematopoietic stromal cells, their extracellular products, and hematopoietic cytokines. The stromal cells include fibroblasts, endothelial cells, adipocytes, osteogenic precursors, and macrophages. These cells produce a complex array of ECM molecules, among others the GAGs, and collagens. The growth, differentiation, and survival of hematopoietic stem/progenitor cells is regulated in the hematopoietic microenvironment by different mechanisms e.g. interactions of hematopoietic progenitor cells with hematopoietic cytokines in association with GAGs or interactions between hematopoietic and stromal cells by means of cell adhesion molecules.
**Hyaluronan (HYA)**

HYA, earlier hyaluronic acid, named after the site where it was found in the vitreous body of the eye (hyaloid=vitreous=glassy) was discovered in the 1930s by Meyer and Palmer (Meyer et al 1934).

HYA, a member of the GAG family, is an ubiquitous anionic polymer with an average length of approximately 25 000 disaccharide units and a high molecular mass >1x10^6. GAGs attached to a protein core are called proteoglycans. In contrast to the other GAGs, HYA is nonsulfated and is lacking a peptide core (Table I). The GAGs, of which HYA is the most abundant, are of utmost importance for tissue structure and function. HYA has been shown to account for 40% of the GAGs in the ECM (Clark et al 1995, Wight et al 1986).

Table I

<table>
<thead>
<tr>
<th>Name</th>
<th>Constituent sugars</th>
<th>Sulphate</th>
<th>Approx.  $M_n$</th>
<th>Proteoglycans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyaluronan</td>
<td>Glucuronic acid Glucosamine</td>
<td>-</td>
<td>10^5 - 10^7</td>
<td>-</td>
</tr>
<tr>
<td>Chondroitin 4-(6-) Sulphates</td>
<td>Glucuronic acid Galactosamine</td>
<td>+</td>
<td>10-50 x 10^3</td>
<td>+</td>
</tr>
<tr>
<td>Dermatan sulphate</td>
<td>Iduronic acid Galactosamine</td>
<td>+</td>
<td>10-50 x 10^3</td>
<td>+</td>
</tr>
<tr>
<td>Keratan sulphate</td>
<td>Galactose Glucosamine</td>
<td>+</td>
<td>5-15 x 10^3</td>
<td>+</td>
</tr>
<tr>
<td>Heparan sulphate</td>
<td>Glucuronic and iduronic acid Glucosamine</td>
<td>+</td>
<td>10-50 x 10^3</td>
<td>+</td>
</tr>
<tr>
<td>Heparin</td>
<td>Glucuronic and iduronic acid Glucosamine</td>
<td>+</td>
<td>5-20 x 10^3</td>
<td>+</td>
</tr>
</tbody>
</table>

HYA exhibits unusual physicochemical properties because of a combination of its random-coil structure, its large size which results in molecular entanglement and its capacity to interact with water molecules. HYA has a large hydrodynamic volume and forms solutions with high viscosity and elasticity that provide space filling, filtering functions and lubricating functions (Balazs et al 1986, Fraser et al 1997, Tammi et al 2002) (Fig 3).
HYA is synthesized in most kinds of vertebrate cells at some point in their natural history. The highest concentrations are found in rooster comb, human umbilical cord and human synovial fluid (Engström-Laurent et al 1985b, Reed et al 1988) (Table II). In the early 1980s methods were developed to measure HYA levels in the range of ng per ml in various body fluids such as serum, urine, pleura fluid and lymph. Few studies have reported on serum concentrations of HYA in patients with haematological diseases. There are reports on elevated serum HYA concentrations in patients with active myeloproliferative disease and multiple myeloma (Hasselbalch et al 1991, Dahl et al 1999). It is also possible to localise HYA histochemically in tissues. Such studies have been performed on animals and humans (Tengblad 1979, Hellström et al 1990, Laurent et al 1991, Laurent et al 1995). Only a few studies have demonstrated the localisation of HYA in bone marrow (Smadja-Joffe et al, Sundström et al 2002, Sundström et al 2005).
Table II

The concentration of sodium hyaluronate in some tissues and body fluids.

<table>
<thead>
<tr>
<th>Tissue/Fluid</th>
<th>Conc. mg/l</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human umbilical cord</td>
<td>4100</td>
<td>Meyer FA et al., 1976</td>
</tr>
<tr>
<td>Normal rooster comb</td>
<td>7500</td>
<td>Szirmai, 1966</td>
</tr>
<tr>
<td>Human synovial fluid</td>
<td>1420-3600</td>
<td>Sundblad, 1965</td>
</tr>
<tr>
<td>Bovine nasal cartilage</td>
<td>1200</td>
<td>Laurent and Tengblad, 1980</td>
</tr>
<tr>
<td>Human vitreous body</td>
<td>140-338</td>
<td>Balazs, 1965</td>
</tr>
<tr>
<td>Human dermis</td>
<td>20</td>
<td>Pearce and Grimmer, 1970</td>
</tr>
<tr>
<td>Human amniotic fluid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>At 16 weeks</td>
<td>20</td>
<td>Dahl et al., 1983</td>
</tr>
<tr>
<td>At term</td>
<td>1</td>
<td>Dahl et al., 1983</td>
</tr>
<tr>
<td>Human urine</td>
<td>0.2</td>
<td>Laurent and Tengblad, 1980</td>
</tr>
<tr>
<td>Human serum (healthy young adults)</td>
<td>0.035</td>
<td>Engström-Laurent, 1985</td>
</tr>
</tbody>
</table>

In bone and cartilage it is probable that HYA turnover occurs by metabolic degradation in situ concurrently with that of collagen and proteoglycans. In skin and joints some HYA is probably turned over by local metabolism, the rest is removed by the lymphatic vessels to the circulation where most is eliminated in the liver sinusoidal cells. As the bone marrow is part of the reticuloendothelial system (RES) it is likely that locally synthesized HYA is at least partly degraded within the bone marrow. The tissue half-life ranges from half a day to two or three days regardless of its route of elimination. (Fraser et al 1984, Fraser et al 1997).

Studies have indicated that during embryonic development the ECM that surrounds migrating and proliferating cells is enriched in HYA, and that during the cell differentiation the HYA concentration is decreased (Toole 1997).

HYA is traditionally regarded as a biological glue that participates in lubricating joint or holding together gel-like connective tissues. Although these are common physiological...
properties of HYA in humans, HYA also has a role in the microenvironment to co-regulate cell behaviour during embryonic development, healing processes, inflammation and tumour development. The association of HYA with tumorigenesis has been known for some time. Several studies have reported a relation between HYA content and invasiveness, and a greater enrichment of HYA in the stroma that surrounds tumours than in the parenchymal regions (Knudson 1989, Delpech et al 1997, Toole 2004).

Recent studies have shown that HYA appears to be critical for the spatial distribution of the transplanted stem cells in vivo and also that the binding of HYA with a surrogate ligand, biotinylated hyaluronic acid-binding protein (HABP) inhibits the proliferation and differentiation of these cells (Nilsson et al 2003).

HYA is not only a simple viscous, space-filling GAG. It also has the ability to activate intracellular signaling cascades and therefore requires interactions with cell-associated HYA-binding proteins called hyaladherins. The first isolated hyaladherin was the receptor of hyaluronan-mediated motility (RHAMM), now designated CD168. RHAMM has recently been identified as the major HYA-binding receptor expressed by mobilized blood hematopoietic progenitor cells (Pilarski et al 1999). Later CD44, another HYA-receptor, was identified. CD44 is now the best understood HYA-receptor. It binds on hematopoietic precursor cells and is highly expressed on stromal cells. There are reports that ligation of CD44 with specific anti-CD44 monoclonal antibodies or with HYA, its natural ligand, can reverse blockage of differentiation in human myeloid leukaemia. These results indicate new possibilities for the development of CD44-targeted differentiation therapy (Turley et al 2002, Mc Donald et al 2002, Charrad et al 1999). There are other HYA-binding receptors identified, e.g. Stabilin-1 and-2, but less is known about their function (Politz O. et al. 2002).
The two major structures of connective tissue are the collagens and GAGs. It is well known that myelofibrosis results from increased deposition of different collagens.

The role of HYA in fibrosis has been discussed. It has been shown that HYA is required for fibroblast detachment and mitosis (Prehm 1984). HYA synthesis has also been found to correlate positively with migration in both foetal and adult fibroblasts (Chen et al 1989). As mentioned before the growth factors PDGF, EGF, TGF-\(\beta\) and bFGF, regulating these cells have been reported to stimulate the synthesis of HYA in cell culture (Shimbukuru et al 2005).

**Classification of haematological and lymphoid neoplasms**

The World Health Organization (WHO) classification of haematological and lymphoid neoplasms has recently been published (Jaffe et al 2001, Vardiman et al 2002). The WHO classification of myeloid neoplasms includes many of the criteria of the French-American-British (FAB) Cooperative Group classifications of acute myeloid leukaemia (AML) (Bennett JM et al 1976), and myelodysplastic syndromes (MDS) (Bennett et al 1982), as well as guidelines of the Polycythemia Vera Study Group (PVSG) for the chronic myeloproliferative diseases (CMPDs) (Berlin 1975, Murphy et al 1997). For the lymphoid neoplasms the WHO classification provides refinement of the entities in the Revised European-American Lymphoma (REAL) classification, a system that is now widely used by pathologists and clinicians (Harris et al 1994).

The WHO classifications of haematological malignancies stratifies neoplasms primarily according to lineage: myeloid, lymphoid, histiocytic/dendritic cell and mastcell. Within each category, distinct diseases are defined according to a combination of morphology,
immunophenotype, genetic features, and clinical syndromes. For each neoplasm, a cell of origin is postulated. Many haematologic malignancies may originate in early precursor cells, and the specific genetic abnormality could determine stage or stages to which the neoplastic cells will differentiate. On the other hand some neoplasms may truly arise in a later cell stage, such as follicle centre cell, in which physiologic gene rearrangements and mutations create a substrate in which tumour-promoting genetic events can occur. The nomenclature is still imperfect and will certainly change in the future as our understanding improves.

**Acute myeloid leukaemia (AML)**

AML refers to a mixture of distinct diseases that differ with regard to their pathogenetic evolution, genetic abnormalities, clinical features, response to therapy, and prognosis. Morphologic, cytogenetic and molecular analyses have been tools to identify disease entities among the different AML types. In the WHO classification, the blast threshold for the diagnosis of AML is 20% blasts in the blood or marrow. In addition, patients with the clonal, recurring cytogenetic abnormalities t(8;21)(q22;q22), inv(16)(p13q22) or t(16;16)(p13;q22) and t(15;17)(q22;q12) should be considered to have AML regardless of the blast percentage. Besides cytogenetic status there are three independent prognostic factors for treatment decisions in *de novo* AML: age, high white cell counts, and the rapidity of attaining complete remission (CR). Unfortunately this has so far not led to major improvements in disease-free and overall survival of adults with this disease (Appelbaum et al 2001, Löwenberg et al 1999). Only about 40% of adult patients with AML between 18-60 years are cured (Büttner et al 2002). AML is a disease of older adults and for the older patients very little progress has been made in long-term survival. The median age of patients with AML is 71 years in Sweden (Swedish Blood Cancer Registry 2004). Intensive chemotherapy with full
postremission therapy is very risky in the elderly, who often suffer from co-morbidity and AML sub-types with unfavorable prognostic factors.

Treatment decisions must be based on individual patient preferences after an informed discussion has taken place that incorporates risk estimates modified to the patients performance status, comorbidities, and leukemia-specific risk factors without using absolute cut-offs for prognostic factors or chronological age.

The treatment in adults with AML classically involves separate treatment phases. The first phase consists of induction chemotherapy, standard regimen anthracyclines for 3 days and cytarabine 7 days, aiming at substantial reduction of the malignant cells and to allow repopulation of the marrow with normal cells, thereby coming to complete remission, defined as <5% blast cells in the marrow. It is clear that once remission is achieved additional therapy is required. The second phase, consolidation, aims to eradicate or reduce the burden of leukemic cells to a level low enough to make cure possible. Usually, these regimens have been based on intensive additional cycles of chemotherapy sometimes followed by autologous or allogeneic hematopoietic stem cell transplantation.

Specific therapies have proven beneficial for small subsets of patients defined by recurring cytogenetic abnormalities. Acute promyelocytic leukemia (APL) represents approximately 10-15% of the AMLs in adults and deserves special attention (Douer et al 1996). With current therapy, including all-trans retinoic acid (ATRA) and anthracycline-based induction, anthracycline-based consolidation and maintenance, 70-80% of patients are alive and free of disease at 5 years (Fenaux et al 1993). The disease is associated with unique genetic features including the t(15;17) and the formation of the PML-RARα fusion transcript. The fusion
transcript permits precise diagnosis and provides the marker for the identification of minimal residual or recurrent disease. Insights into the mechanism of leukemogenesis and resistance in APL serve as a paradigm for other AMLs. Treatment with ATRA demonstrates that the novel strategy of differentiation therapy can be highly effective. APL was once characterized by a high early death rate, but has now become the most curable subtype of AML.

A goal for many researchers over the past decades has been to create an effective immunologic approach to the treatment of AML. CD33 is a glycoprotein found on the leukemia cells in more than 90% of the AML patients. Unconjugated antibodies to CD33 do not have major clinical activity in overt AML. This observation together with the fact that CD33 is absent from the surface of normal hematopoietic stem cells and all non-hematopoietic sites suggested that antibodies to CD33 might serve as an effective vehicle to target potent drug conjugates to leukemic cells while sparing the normal hematopoietic stem cell. The drug gemtuzumab ozogamicin (Mylotarg), which joins a humanized anti-CD33 IgG4 antibody to a potent antitumor antibiotic calicheamicin, is one example of a therapy based on an immunologic approach (Sievers et al 1999). So far the drug has mostly been used for older patients in first relapse and now there are ongoing trials using the drug in combination with chemotherapy in older patients with de novo AML and also in younger patients in remission as maintenance prior to nonablative transplantation.

In the last decade investigators have begun to focus on allogeneic stem cell transplantation as a potential immunotherapeutic approach rather than primarily as a vehicle for delivering high dose therapy.
Hematopoietic stem cell transplantation is an effective therapy for AML. Obstacles to broad applicability of stem cell transplantation for the majority of the patients have included inability to control leukemia with induction therapy, lack of suitable hematopoietic stem cell donor, toxicities of conditioning regimens and post transplant complications. All antileukemia therapies are complicated with the problems of chemotherapy-related toxicities and disease relapse.

Whether autologous stem cell transplantation (autoSCT) following high-dose cytotoxic therapy is better than intensive chemotherapy has been discussed during the past decade. Several large prospective studies were designed. However, the results have been confusing and the data difficult to interpret. For example the question whether autoSCT might benefit particular prognostic subgroups has not been settled (Zittoun et al 1995, Burnett et al 1998, Cassileth et al 1998, Harrousseau et al 1997).

Allogeneic stem cell transplantation (alloSCT) compared to autoSCT also exposes the patient to the risks of organ toxicities caused by graft-versus-host-disease (GVHD) and prolonged immunosuppression with its attendant risks of post transplant infectious complications. The potential power of the graft-versus-leukemia (GVL) effect coupled with the ability to achieve allogeneic engraftment without the toxicities associated with very high dose therapy has generated increased interest in transplantation with reduced intensity conditioning as an immunotherapeutic step.

Little is known about the prevalence and importance of myelofibrosis in the AML subgroups, except for acute leukaemia with megakaryocyte differentiation. One reason is that estimation of fibrosis requires bone marrow biopsy and the diagnosis in AML can easily be made from
bone marrow aspirates. Biopsy has usually been performed only in case of dry tap. Thus the evidence of fibrosis in AML is not usually adequately documented and the concept that myelofibrosis in AML is a poor prognostic factor, and may be a sign of relapse, has still to be proved (Manoharan et al 1979, Thiele et al 1991, Islam 1993). In our study we found a lower, but normal, score of reticulin and a higher grade of HYA in AML patients at diagnosis, compared to a control group. However in the subgroup of patients with MDS the reticulin score was higher compared to the other AML patients (Sundström et al 2005). This is an interesting finding although the origin and cause of the increased HYA content are unknown.

**Chronic myeloproliferative diseases (CMPDs)**

The first report of patients with idiopathic myelofibrosis was published by Heuck in 1879, who described two patients with severe bone marrow fibrosis and extramedullary hematopoiesis in the liver and the spleen.

Polycythemia vera, essential thrombocythemia, idiopathic myelofibrosis, and chronic myelogenous leukaemia are the entities traditionally classified as CMPDs (Dameshek 1951). The recent World Health Organization classification (Jaffe et al 2001) of the CMPDs includes polycythemia vera (PV), essential thrombocythemia (ET), chronic idiopathic myelofibrosis (CIMF), chronic myelogenous leukaemia (CML;Philadelphia chromosome, t(9;22)(q34;q11), BCR/ABL pos), chronic neutrophilic leukaemia (CNL), chronic eosinophilic leukaemia (CEL) and chronic myeloproliferative disease, unclassifiable (CMPD,U). All CMPDs are clonal haematopoietic stem cell disorders characterised by proliferation in the bone marrow of one or more of the myeloid lineages i.e. granulocytic, erythroid and megakaryocytic. CNL and CEL are very uncommon diseases.
The CMPDs are primarily diseases of adults with peak in frequency in the fifth and seventh decades of life. The incidence for all CMPDs is approximately 6-9/100 000 population per year (Mesa et al 1999). The complications these patients meet with in different degrees are anemia, infection, trombohaemorrhagic episodes, extramedullary hematopoiesis and splenohepatomegaly.

**CML**

CML is the most common of the CMPDs and holds an exceptional position among these disorders. It was first described in the nineteenth century (Bennet 1845, Neumann 1878). In 1960 an abnormal chromosome was documented in leukemia cells from CML patients (Nowell et al 1960). The new marker was named Philadelphia chromosome (Ph) in honor of the city where it was discovered. This was the first consistent chromosome abnormality documented in human malignancy. In the 1970s it was demonstrated that the Ph-chromosome was a translocation between chromosome 9 and 22, t(9;22)(p34.1;q11.21) (Rowley 1973). In the 1980s investigators showed that the ABL protooncogene on chromosome 9 was reciprocally translocated near the BCR gene on chromosome 22, resulting in BCR-ABL proteins. An abnormal fusion protein is formed which has tyrosine kinase activity and is overexpressed in cells from CML patients (Wada et al 1995).

CML is characterized by two distinct clinical phases. The first one, chronic phase is marked by proliferation of myeloid cells showing full range of maturation. Symptoms of vigorous hematopoiesis and/or extramedullary hematopoiesis like fever, sweats, bone pain, weight loss, and fatigue or signs from splenomegaly lead the patients to the doctor. More unusual symptoms are thrombosis, bleeding, priapism. Later a decrease in the myeloid differentiation
generally occurs and the disease enters an advanced stage, accelerated phase, which precedes blast crisis with poor prognosis, characterized with all the manifestations of acute leukaemia. As the other CMPDs, CML originates in abnormal bone marrow pluripotent stem cells. The growing understanding of the BCR/ABL proteins has led to new diagnostic tests and new therapies which make it possible to cure CML patients. Imatinib (Gleevec) is a new drug, a specific tyrosine kinase inhibitor, which inhibits the proliferation of CML cells by inhibiting BCR/ABL kinase activity. Treatment with imatinib can result in cytogenetic remission, and even molecular remission, but the duration of the response is not known. Drug resistance to imatinib occurs and research continues to find new treatments and combinations of treatments. Allogeneic stemcell transplantation, in a selected group of patients, is at present the only treatment that gives a long-term leukaemia-free survival (Elmaagacli et al 2002, Martino et al 2001). Before the era of imatinib different single-agent chemotherapies have been used to control the chronic phase of CML e.g. hydroxyurea, busulfan and α-interferon and combinations of these. α-Interferon is the only of these drugs that has shown complete cytogenetic response and may also prevent fibrosis, but the response rate is considerably lower than with imatinib (Silver et al 1999, Wilhelm et al 1998).

Myelofibrosis is a complication in CML. It is known that myelofibrosis is an unfavourable prognostic factor at diagnosis and that a subtype of CML patients with megakaryocytic proliferation present a "pre-myelofibrotic" stage (Dekmezian et al 1987, Thiele et al 1991, Kvasnicka et al 2001, Buhr et al 1992). There are conflicting reports about which treatment, with the exception of allogeneic transplantation, can stop or reverse fibrosis. A study of repeat bone marrow biopsies from CML patients treated with either hydroxyurea, busulfan or interferon showed the evolution of fibrosis and found that only patients treated with hydroxyurea displayed regression of myelofibrosis. In patients treated with busulfan and
interferon there was a progression (Thiele et al 2000). Other studies have described no
effect enhancing but rather a preventing effect of fibrosis on patients treated with α-interferon

PV, ET, CIMF

PV, ET and CIMF are disorders sharing some common features. They are clonal
haematopoietic stem cell disorders characterised by proliferation of one or more of the
myeloid lineages. Hypercellularity in the bone marrow and increased numbers of
granulocytes, red blood cells and/or platelets in blood as well as megakaryocyte dysplasia and
hyperplasia are seen. Splenomegaly and hepatomegaly are often found and are caused by
excess of blood cells, extramedullary haematopoiesis, leukaemia infiltration, or any
combination of these in the spleen or liver. Spontaneous growth in vitro of erythroid and/or
megakaryocyte progenitor cells is characteristic for PV and is also seen in a proportion of
patients with ET and CIMF (Lacombe et al 1980, Kralovics et al 2003). Transformation to
acute leukaemia occurs in PV, ET and CIMF, but at a lower rate compared with the rate in
CML. It has been discussed whether this depends on the type of treatment or the clonal
disease itself (Berk et al 1981, Kaplan et al 1986). Fibrosis is an important bone marrow
finding overlapping the various CMPDs. Most of the CIMF patients (70-80%) are initially
diagnosed in the fibrotic stage. In PV, marrow fibrosis occurs in about 30%, whereas fibrosis
is rarely seen in ET patients.

In patients with PV the main cause of mortality and morbidity is thrombosis and rather often
bleeding. There is also a variable incidence of progression to myelofibrosis or AML. The
cornerstone in treatment of PV is phlebotomy, to reduce the red cell mass to maintain
hematocrit <0.45. If poor compliance to phlebotomy, high risk of thrombosis or progressive myeloproliferation, indicated by increasing splenomegaly or high leukocyte or platelet counts, cytoreductive treatment is recommended. Hydroxyurea is the most frequently used drug. Reduction of fibrosis during treatment with hydroxyurea has been reported (Löfvenberg et al 1990). Studies have shown reduction of thrombosis and fibrosis in patients treated with hydroxyurea compared with patients only treated with phlebotomy (Fruchtman et al 1997, Tartarsky et al 1997). Interferon is not a cytotoxic agent with the advantage of not being the cause of secondary malignancy and an alternative for younger patients although the side effects are not negligible. Busulfan and P32 are conceivable drugs, but they have a mutagenic potential and are therefore only recommended in the elderly (Finazzi et al 2005). Anagrelide, a non-cytostatic drug, can be considered to control thrombocytosis but not myelofibrosis (Anagrelide study group 1992, Thiele et al 2003). Low-dose aspirin can safely prevent thrombotic complications in patients with PV who have no contraindications to such treatment (Landolfi et al 2004). Allogeneic stem cell transplantation has been used in a few selected patients and some have been cured while autologous stem cell transplantation has only a palliative effect (Jurado et al 2001, Anderson et al 2001).

**ET** is an entity in which an excessive, persistent number of morphologically and functionally abnormal platelets are produced. The diagnosis of ET also takes into account the number and size of the megakaryocytes, which are increased. There is no Philadelphia chromosome, no increase of red-cell mass and no collagen fibrosis. (Kutti 1990, Kutti et al 1996, Tefferi et al 2001). The clinical features of ET are events of thrombosis and to a lower extent events of hemorrhage, particularly if the platelet count is very high. High age, a previous thrombotic event, and long duration of thrombocytosis are risk factors for thrombosis in ET (Cortelazzo et al 1990, van Genderen et al 1994, Lengfelder et al 1998). ET may transform to
myelofibrosis and AML (Cervantes et 2002, Andersson et al 2000). Treatment of ET patients should be based primarily on the expected risk of thrombotic complications. The concept of risk-stratified management is generally accepted in ET but the criteria used are still under debate. Low risk patients up to 60 years, without prothrombotic comorbidity, with platelets <1000-1500x10^9 are recommended no cytoreductive therapy but low-dose aspirin for patients with no contraindication to aspirin (Barbui et al 2004). A randomized MRC PT1 (Medical Research Council Primary Thrombocythaemia 1) study has recently shown that for patients at high risk for vascular events hydroxyurea plus low-dose aspirin is superior to anagrelide plus low-dose aspirin. Arterial thrombosis, major haemorrhage and myelofibrosis were more frequent for the patients treated with anagrelide. Venous thrombosis was paradoxically more frequent in those treated with hydroxyurea. Both hydroxyurea and anagrelide can control platelet count but this seems not to be a reliable indicator to reduce thrombotic risks. A treatment option for pregnant women and younger patients is interferon (Harrison et al 2005a, Harrison et al 2005b).

CIMF, also known as agnogenic myeloid metaplasia or nowadays most commonly myelofibrosis with myeloid metaplasia (MMM), is characterized by intense bone marrow stromal reaction including fibrosis, osteosclerosis, and angiogenesis (Tefferi 2000). CIMF can present de novo or develop post PV or ET. The diagnosis is often suspected when teardrop-shaped red cells and myeloid precursors are detected in peripheral blood. The clinical phenotype includes progressive anemia, massive splenomegaly, hepatosplenie and non-hepatosplenic extramedullary hematopoiensis. Hypercatabolic symptoms with fatigue, night sweats, low-grade fever and weight loss are seen in a variable degree. Even severe complications with portal hypertension due to splenomegaly and intrahepatic obstruction in portal veins occur. Some CIMF patients are asymptomatic and need only observation. A
selected group of CIMF patients may be cured by allogeneic hematopoietic stem cell transplantation either myeloablative or reduced intensity, while autologous transplantation primarily is a palliative procedure that slows down the process (Guardiola et al 1999, Devine et al 2002, Anderson et al 1999). Hydroxyurea, busulfan, interferon and cladribine are drugs that can control leukocytosis, thrombocythosis, or organomegaly (Gilbert et al 1998, Tefferi et al 1997). Experimental drug therapies are explored, intended to interfere with cytokines, and believed to mediate the bone marrow fibrosis and angiogenesis e.g. thalidomide or thalidomide analogues with different combinations of prednisone and cyclophosphamide (Mesa et al 2003). Splenectomy can be recommended in some selected cases e.g. with hydroxyurea resistant splenomegaly and splenic pain (Tefferi et al 2000).

Until now the underlying target genes that contribute to the myeloproliferative clone in PV, ET, and CIMF have been elusive. Two aberrations, polycythemia vera rubra 1 (PRV-1) and the ability to form endogenous erythroid colonies (EECs) are highly correlated in some CMPD patients (Kralovics et al 2003, Griesshammer et al 2004). Recently, several publications have shown that mutation in the Janus kinase gene (JAK2V617F) is present in virtually all patients with PV, and in about 50% of ET and CIMF patients. The mutation is not seen in CML patients or in healthy individuals. These results will be important in the future for classification, diagnosis, and hopefully treatment of the CMPDs (Cazzola et al 2005, James et al 2005, Baxter et al 2005, Levine et al 2005, Kralovics et al 2005, Jones et al 2005, Goerttler et al 2005).

**Hairy cell leukaemia**

Hairy cell leukaemia (HCL) is a rare disease, in the middle-aged and elderly adults, initially described in 1958 (Bouroncle et al 1958). It accounts for about 2% of the leukaemias in US
with an incidence of 3.5 per million, five times more men than women (Bouroncle 1994). Most commonly the patients present with pancytopenia and splenomegaly. HCL is defined as a leukaemia with small B-lymphocytes with oval nuclei and abundant cytoplasm with “hairy” projections. The cells are found predominatly in the bone marrow and spleen and in a small amount in the circulation. They are characterised by tartrate resistant acid phosphatase positivity and strongly expressing CD103, CD22 and CD11c. There is an increase of myelofibrosis in HCL, shown with reticulin staining. This increase often results in ”dry tap” at bone marrow aspiration. The diagnosis is therefore best made on bone marrow biopsy (Burke et al 1974). HCL was one of the first malignancies to show complete responses to interferon (Quesada et al 1984). First-line treatment nowadays is a 7-day course with the purine analog 2’-Chlorodeoxyadenosine (cladribine), which is highly effective. Complete and durable remission is observered in 75-90% of treated patients (Estey et al 1992, Juliusson et al 1992, Beutler 1994).
AIMS

The aims of the present studies were:

* To study the localisation and distribution of HYA in the bone marrow in healthy adults and correlate HYA with reticulin staining, the established way to quantify myelofibrosis. Paper I.

* To compare two fixation methods, A and B, and see whether we could find any differences in morphology and quality of HYA and reticulin staining. Method A is an improved technique for HYA histochemistry using microwave irradiation. Method B is the routine method used in the Department of Pathology at our hospital. Paper I

* To develop a grading scale to evaluate the HYA staining intensity in bone marrow. Paper I

* To describe the HYA intensity, compared with reticulin staining, in the bone marrow of patients with various hematological disorders compared with the control group of healthy individuals. Paper II, III and IV.

* To investigate the effect of anagrelide treatment on bone marrow fibrosis in patients with CMPDs, estimated with HYA and reticulin staining. Paper IV.
MATERIAL AND METHODS

Paper I.

Bone marrow trephine biopsies were collected from crista iliaca posterior from 30 healthy volunteers compared with biopsies from 3 patients with known myelofibrosis. The biopsies were cut into two pieces and processed according to two fixation-methods, A and B. Method A is an improved technique for HYA histochemistry using microwave irradiation (Tengblad 1979 and Hellström et al 1990). Method B is the routine method used in the Department of Pathology at our hospital. These fixation methods were compared with respect to differences in morphology and quality of staining. The sections, irrespective of fixation method, were stained with routine hematoxylin-eosin for evaluation of morphology, silver impregnation for visualization of reticulin fibre according to Laidlaw (Putt 1972, Bancroft et al 1982), and hyaluronan binding protein (HABP) for histochemical localisation of HYA (Tengblad 1979, Hellström et al 1990). All sections were evaluated blindly by one pathologist (I.H.).

Method A. Within less than 20 minutes after the biopsy had been taken one part of the bone marrow specimen was transferred from saline to a solution of 2% formalin and 0.5% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS) pH 7.35 and fixed in a Bio-Rad H 2500 MW processor at a setting of 45°C using 700 W. Decalcification was performed by putting specimens in a 10% EDTA solution and processing them in a microwave oven at 75% effect for between 4 and 6 hours. Microwave-fixed and decalcified samples were dehydrated in ethanol and embedded in paraffin. Serial sections were mounted on glass slides.
Method B. The other bone marrow piece was transferred from saline to a fixation solution of buffered formalin consisting of 68 g methanol, 8 g 4% formaldehyde and 4 g concentrated acetic acid. The bone marrow piece was decalcified for 10 minutes in Decalc®, neutralised in phosphate buffer for 6 hours, dehydrated, and embedded in paraffin. Serial sections were mounted on glass slides.

Isolation and biotin labelling of HABP.

The isolation and biotin labelling of the HABP has been described by Tengblad 1979 and Hellström et al 1990. Briefly, a mixture of proteins with affinity for HYA was isolated from bovine nasal cartilage and purified by affinity chromatography. The purified HYA binding region was then linked to biotin and stored at -20°C until used. The HABP was a kind gift from Pharmacia AB, Uppsala Sweden.

Histochemical staining for HYA.

After deparaffinisation, the sections were rinsed in PBS, incubated with fresh solution of 3% H₂O₂ in methanol for 5 min at room temperature to inhibit any endogenous peroxidase activity, and rinsed twice in PBS. The slides were then incubated with 1.0% bovine serum albumin for 30 min at room temperature to block non-specific binding sites, and washed in PBS. Control slides were incubated with 50 U per mL Streptomyces hyaluronidase (Sigma, St Louis, Mo., USA) for 4 h at 37°C. This hyaluronidase specifically degrades HYA and therefore serves as a control, to confirm specificity. All slides were then rinsed with PBS, 2x10 min, and incubated at 4°C, with about 100 μL of HABP, at a dilution of 1:40 overnight, rinsed in PBS,2x10 min, then incubated with avidin-biotin complex (ABC) reagent, 1:200 dilution (Vector Laboratories, Burlingame, Calif., USA), for 40 min at room temperature. After three more 10 min rinses in PBS, the sections were incubated for 5 min in 0.05%
diaminobenzidine tetrahydrochloride (Sigma, St. Louis, Mo, USA) and 3% H₂O₂ in 0.05% TRIS-HCL buffer, pH 7.6, at room temperature, producing a water-insoluble brown precipitate. Finally, the slides were rinsed in tap water for 5 min and cover-slipped.

*Grading of bone marrow reticulin.*

The bone marrow biopsies were stained with silver impregnation for visualization of reticulin fibres and scored (Bauermeister 1971) on a six graded scale: 0, non reticulin fibres demonstrable; N, occasional fine individual fibres only; 1+, occasional fine individual fibres plus foci of fine fibre network; 2+, fine fibre network throughout most of the section with no coarse fibres demonstrated; 3+, diffuse fibre network with scattered thick, coarse fibres but no true collagen; 4+, diffuse, often coarse, fibre network with areas of collagenization. According to Bauermeister the upper normal limit for bone marrow biopsies is 2+.

*Grading of HYA staining intensity.*

Bone marrow content of HYA was evaluated according to the intensity of the staining. The HYA staining was evaluated on a four-grade scale: 1, sparse; 2, weak; 3, moderate; 4, intense. 1, sparse and 2, weak were considered normal.

*Comparison of method A and B.*

Fixation method A and B were compared concerning differences in morphology and quality of HYA and reticulin staining. The two fixation methods showed no differences.

**Paper II**

In collaboration with professor Rie Dahl, at Section of Haematology at the University of Tromsö, 61 bone marrow biopsies were obtained from patients with different malignant
diseases. Forty-four of these patients had a disease involving the bone marrow; one biopsy came from a patient with CML, 2 B-cell lymphoma not otherwise specified (NOS), 1 T-cell lymphoma, 7 AML, 3 ALL (acute lymphoblastic leukaemia), 14 MDS, 6 myeloma, 3 chronic lymphocytic leukaemia (CLL), 4 hairy-cell leukaemia (HCL), and 3 Mb Waldenström.

Seventeen of the patients had a malignant disorder not involving the bone marrow; one biopsy from a patient with bladder cancer, one prostate cancer, 6 B-cell lymphoma NOS, 1 T-cell lymphoma NOS, 2 mantle cell lymphoma, 2 diffuse large B-cell lymphoma (DLBCL), 2 Hodgkin lymphoma, 2 plasmacytoma.

The same staining and scoring methods for HYA and reticulin were used as in paper I. The HABP was a kind gift from Pharmacia AB, Uppsala. All samples were coded and examined blinded by one pathologist (M.H.), the same as in paper III and IV.

As there was one pathologist (I.H.) in paper I and another pathologist (M.H.) in paper II-IV, the latter reexamined, coded and blinded, the bone marrow sections analysed in paper I. The HYA and reticulin staining agreement was 93% respectively 85%, with a difference of one score.

**Paper III**

Bone marrow trephine biopsies were collected from the posterior iliac crest from 35 patients with newly diagnosed AML, before start of antileukaemic treatment. Fixation method B was used as described in paper I. Staining and grading for HYA and reticulin were done as described in paper I. HABP was a kind gift from Pharmacia AB, Uppsala. All samples were coded and examined blinded by one pathologist (M.H.), the same as in paper II and IV.
**Paper IV**

A multicenter, open, prospective, phase II study of anagrelide treatment in 60 patients with thrombocythemia due to CMPD was performed by the Swedish Myeloproliferative Disorder Study Group. Bone marrow trephine biopsies were collected at start of the study and adequate samples were obtained in 53 patients. Biopsies were taken before start of anagrelide treatment, after 6 months, and after 2 years on treatment. At the end of the study there were 19 patients with adequate biopsies both from start and at 2 years. The cellularity, amount of megakaryocytes and staining for HYA and reticulin were evaluated. Biopsies from 53 of the 60 CMPD patients at start were available and compared with the biopsies from the 30 healthy volunteers described in paper I and the 34 patients with AML described in paper III. Hematoxylin-eosin stained sections were used for evaluation of morphology, cellularity, and number of megakaryocytes/mm. At least four marrow spaces with high quality had to be present in a biopsy for inclusion and evaluation. The same staining and scoring methods for HYA and reticulin were used as in paper I. All samples were coded and examined in a blinded fashion by one pathologist (M.H), the same as in paper II and III. The HABP was a kind gift from Corgenix Co, USA. This HABP was compared with the HABP from Pharmacia AB Uppsala, used in paper I, II and III and was found to have the same staining quality.

**Statistics**

Independence between HYA and reticulin staining and differences between sexes according to histological scoring, in the control group, were calculated with Pearson’s chi-square test. Differences in age distribution between HYA and reticulin scores were tested with Kruskal-Wallis test. Paper I.
The HYA and reticulin grading were compared between the controls and the patients with a malignant disease with and without bone marrow involvement, using Kruskal-Wallis and Mann-Whitney U test. The relation between HYA and reticulin grading was analysed using Pearson’s chi-square test. Paper II.

The HYA and reticulin grading in the AML patients were compared with the control group and tested with Pearson’s chi-square test. The same test was used for calculating the relation between clinical and laboratory variables and HYA and reticulin grading scores. Paper III.

The reticulin and HYA grading, cellularity and number of megakaryocytes/mm² in the consecutive bone marrow samples were compared using Wilcoxon signed ranks test. The reticulin and HYA grading were compared between normal controls, AML and CMPD patients using Kruskal-Wallis and Mann-Whitney U test. The relation between reticulin and HYA grading was analyzed using Pearson’s chi-square test. Paper IV.

Differences were considered significant when the $p$-value was < 0.05

**Ethics**

The studies were approved by the Ethics Committee at Umeå and Uppsala University and performed in accordance with the Helsinki Declaration of 1975, revised in 1983.
RESULTS AND DISCUSSION

We have reported a correlation between HYA and reticulin staining in the bone marrow in a control group of healthy individuals, in patients with *de novo* AML and in patients with different malignant diseases with and without bone marrow involvement. We have also found that both HYA and reticulin scores in patients with various malignant disorders involving the bone marrow were higher compared both to patients with malignant diseases without bone marrow involvement and to the control group. Fibrosis is an important bone marrow finding in CMPD patients. One of the aims with cytoreductive treatment of these patients is to keep fibrosis under control. In one of our studies we followed CMPD patients during two years treatment with anagrelide and found that HYA, reticulin and cellularity increased, indicating progression of fibrosis, which means that anagrelide is not the drug of choice for reduction of fibrosis.

The established method to evaluate myelofibrosis is reticulin staining. However, the dynamics and velocity of the fibrotic process can not be studied with this method. As collagen fibres are laid out on a surface of HYA, synthesis of this polysaccharide may be the earliest sign of impending fibrosis. Future prospective studies, including larger numbers of patients, investigating HYA and reticulin deposition for evaluation of fibrosis could give an answer to the advantage of using both reticulin and HYA or HYA alone as a marker of fibrosis.
CONCLUSIONS

- HYA occurs in normal bone marrow, demonstrated in the controls. A four-grade scale evaluating HYA is described. The staining pattern of HYA shows a sparse and uneven distribution in the ECM and a more homogenous pattern in the adventitia of the large vessels. This is concordant with the reticulin staining, the established way to quantify myelofibrosis.

- There is a correlation between HYA and reticulin in normal bone marrow, the variables are not independent.

- In patients with malignant disease involving the bone marrow, HYA staining is more pronounced compared to the patients without involvement in the bone marrow and the controls. The reticulin grading is higher in the patients with the disease involving the marrow compared to those without involvement in the bone marrow and to the controls. In all these patients there is a correlation between HYA and reticulin as is seen in the controls. This maybe a sign of impending fibrosis and/or tumour activity.

- In the patients with de novo AML the deposition of HYA is increased. The reticulin score is normal but lower, compared to the controls. The subgroup with antecedent MDS has a higher reticulin score compared to the other AML patients. There is a correlation between HYA and reticulin in the bone marrow of the AML patients, as is seen in the patients with other malignant diseases with and without bone marrow involvement, and in the controls.

- Reticulin and HYA scores are higher in the CMPD patients, before anagrelide treatment, compared both to the AML patients and the controls. After two years treatment of the CMPD patients the reticulin and HYA scores as well as the cellularity are higher than before treatment, but not the number of megakaryocytes.
• The increase of HYA and reticulin in the bone marrow after two years of treatment with anagrelide indicate progression of fibrosis. Anagrelide is unable to stop progression of fibrosis and hypercellularity.

• Fixation method A, using microradiation described for HYA histochemistry, is compared with fixation B, the routine fixation method used in the Department of Pathology in our hospital. No difference between the two methods is observed. Any retro-and prospective HYA study can therefore be performed on bone marrow biopsies processed according to method B.
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