ABSTRACT

The chronic myeloproliferative disorders, comprising polycythemia vera (PV), essential thrombocytosis (ET), chronic myeloid leukaemia (CML) and myelofibrosis (MF), are all characterized by pathological angiogenesis in variable degrees, e.g. increased microvascular density (MVD) and bizarre vessel architecture. The aim of this study has been to characterize the molecular background to this phenomenon by asking two questions. The first is: is there a specific marker for newly formed vessels in the bone marrow of patients with myeloproliferative disorders? The second is: what is the mechanism responsible for the abnormal vessel morphology seen in these disorders?

To answer the first question, a panel of antibodies, known to identify antigens over-expressed on endothelial cells of solid tumors (Tie-2, Cox-2, endoglin, angiomodulin and glycodelin) was used to stain bone marrow biopsies from patients with PV, CML and controls. None of these markers were over-expressed in these patients, suggesting that the vessel phenotype in myeloproliferative disorders is different from that of solid tumors. Surprisingly, we found that Tie-2, Cox-2 and glycodelin were expressed by megakaryocytes in both patients and controls. Since there is an accumulation of megakaryocytes in the bone marrow in myeloproliferative disorders, the total release of angiogenic factors is probably enhanced; thus, this milieu intérieur might be of significance for the pathological angiogenesis.

To reveal the mechanism for the abnormal vessel morphology, we focused on the disorder showing the most aberrant vessels, namely myelofibrosis. VEGF expression and pericyte coverage in the bone marrow were studied and we could show both an over-expression of VEGF as well as increased pericyte coverage of the aberrant vessels. The latter finding was surprising, since pericyte coverage is thought to be a sign of maturity, and decreased pericyte coverage was expected based on findings in solid tumors.

To further define the molecular events orchestrating these events, we studied microvascular density and pericyte coverage in two mouse models of myelofibrosis: mice over-expressing thrombopoietin (TPO) and mice with low expression of the transcription factor GATA-1 (GATA-1^{low}). Both TPO and GATA-1 are important factors in megakaryocyte differentiation, and both mice models show abnormal megakaryocyte maturation. We found that both these models develop morphologically aberrant vessels and increased MVD. Similar to human MF, many of the aberrant vessels were pericyte coated, suggesting that these cells also are involved in the pathological angiogenesis.

By using these mice models we can demonstrate a link between deficient megakaryocyte maturation and pathological angiogenesis. It is, thus, tempting to suggest that the dysmaturation of megakaryocytes seen in human MF is responsible for the increased angiogenesis, but that remains to be proven.

Repro Print AB
ISBN 91-7140-383-3

Prologue

This thesis, titled angiogenesis in myeloproliferative disorders, is the result of my latest efforts in the scientific arena. It is written, as is the custom, in a scientific language, aimed at convincing you of the accurateness and sincerity of the results obtained. However, scientific language has the serious side effect of pulling away some of the drama of the story told, the reader is easily lost in tedious descriptions of methods and results obtained by others. Thus, for all of you who will get this thesis in your hands, not planning to read anything but the acknowledgements, I would just like to present my story in a few words.

The first part of the story (Act I and II) could be titled “the quest of the missing marker”. It describes the perils and adventures encountered on my way trying to find a marker for newly formed blood vessels in the bone marrow. I can reveal that the marker is still elusive, but as in many good stories other wisdoms were learned on the way.

In Act III, the story turns into a romantic piece alternatively titled “the story of the passionate pericyte and the enticing endothelial cell” (cover picture). The setting is the deadly disease myelofibrosis where these two cells, against all odds, are attracted to each other, meet and multiply. In Act IV, “mice and men” the drama continues in a more rodent setting, but no less dramatic. I will not reveal if the end is happy or not, you just have to read for yourselves.

Front cover page: It takes two to tango
Myeloproliferative disorders are characterized by increased angiogenesis and abnormal vessel morphology in the bone marrow. Since it takes two to make a functional vessel: endothelial cells (red color) and pericytes (green color), clues to the pathogenesis of these disorders might be found in either cell type.

LIST OF ORIGINAL PAPERS

This thesis is based on the following papers, referred to in the text by their Roman numerals

I. Zetterberg E, Lundberg LG, Palmblad J. Characterization of blood vessels in bone marrow from patients with chronic myeloid leukemia and polycythemia vera.
Scand J Clin Lab Invest. 2004;64(7):641-7
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II. Zetterberg E, Lundberg LG, Palmblad J. Expression of Cox-2, Tie-2 and glycodelin by megakaryocytes in patients with chronic myeloid leukaemia and polycythaemia vera.
Br J Haematol. www.tandf.no/sjcli 2003 May;121(3):497-9
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III. Zetterberg E, Hoffman A, Rogers R, Dickie R, Palmblad J. Pericyte coverage of abnormal vessels in the myelofibrosis bone marrow.
(Submitted)

IV. Zetterberg E, Vannucchi AM, Migliaccio AR, Wainchenker W, Palmblad J. Angiogenesis in bone marrows of mice with targeted gene manipulations (GATA-1 ^{low} and TPO) leading to myelofibrosis.
(Submitted)

CONTENTS

PPOPULÄRVETENSKAPLIG SAMMANFATTNING

För att en tumör ska kunna växa sig större än 1 mm^2 krävs att tumören börjar bilda ett eget kärlnätverk (angiogenes). I solida tumörer drivs denna kärlnybildning (angiogenes) av att tumörcellerna bildar proangiogenetiska faktorer som stimulerar närliggande endotelceller att bilda nya kärl via knoppning och utväxt. Nya kärl kan också skapas genom att inväxande tumörceller delar upp existerande kärl i flera hålrum, eller genom att förstadier till endotelceller från benmärgen fastnar i tumörkärlet och utvecklas till nya endotelceller.

De nybildade kärlen skiljer sig på en rad olika punkter från normala kärl. De är ofta slingriga, med växlande kaliber och har stora hål i kärlväggen. I vissa tumörer saknas stödjeceller, pericyter, som normalt finns på utmognade kärl. En rad olika faktorer, som reglerar kärlnybildning, har visat sig vara överuttryckta i tumörkärl, men någon faktor som är helt specifik för nybildade kärl har ännu inte säkert kunnat påvisas.

Även vid hematologiska maligniteter som akut leukemi, myelom och lymfom ses en ökad kärlnybildning i benmärg. Den mikrovaskulära densiteten (dvs. antal kärl per synfält i mikroskopet, MVD) är kopplat till sjukdomsförloppet: ju högre MVD, desto aggressivare uppträder sjukdomen. Att mäta MVD i benmärg är dock komplicerat och svårt att standardisera, varför det inte fått något genombrott i klinisk praxis.

Även vid de myeloproliferativa sjukdomarna (polycytemia vera (PV), essentiell trombocytemi (ET), kronisk myeloisk leukemi (KML) och myelofibros, (MF)) ses en patologisk kärlnybildning i benmärg, mest uttalat vid KML och myelofibros. Dessa kärl uppvisar samma formförändringar som kärl i solida tumörer, dvs. de är rikt förgrenade, vindlande och uppvisar kaliberväxlingar.

Målsättningen med mitt doktorandarbete har varit att undersöka de mekanismer som ligger bakom den sjukliga kärl-nybildningen som ses i benmärgen hos patienter med myeloproliferativa sjukdomar. Syftet är att hitta sjukdoms-specifika förändringar mot vilken man i framtiden kan rikta behandling.

I delarbete I var syftet att påvisa en specifik markör för nybildade kärl i benmärg hos patienter med PV och KML. Patientmaterialet infärgades immunhisto-kemiskt med en panel av antikroppar riktade mot faktorer som visat sig vara överuttryckta i kärl i solida tumörer. Vi undersökte uttryck av: Tie-2, endoglin (CD105), Cox-2, angiomodulin och glycodelin. Av dessa var det endast Tie-2 som uttrycktes på endotelceller. För att undersöka huruvida Tie-2 var överuttryckt på nybildade, malignitetsassocierade kärl gjordes dubbelfärgningar med CD34, som identifierar endotelceller, och Tie-2. Majoriteten av kärlen visade sig uttrycka både CD34 och Tie-2, men hos både patienter och kontroller påvisades kärl som bara uttryckte CD34 eller bara Tie-2. Vi fann inga hållpunkter för att Tie-2 skulle vara överuttryckt på kärl hos patienter med PV eller KML. Dessa fynd talar för att de nybildade kärlen vid PV och KML har en annorlunda fenotyp än de i solida tumörer

Då vi studerade uttrycket av de ovan nämnda proangiogenetiska faktorerna: Cox-2, Tie-2 och glycodelin visade de sig något överraskande kunna påvisas i förstadier till blodplättar, megakaryocyter i benmärgen. Då både PV och KML karakteriseras av defekt utmognad av megakaryocyter, undersökte vi i delarbete

II om det förelåg något sjukdomsspecifikt över- eller underuttryck av dessa faktorer. Glycodelin och Cox-2 visade sig vara uttryckt i samtliga megakaryocyter, både hos patienter och hos kontroller, medan uttrycket av Tie-2 var mer variabelt. Någon skillnad mellan patienter och kontroller kunde dock inte påvisas. Vi beskriver i detta arbete det ej tidigare publicerade uttrycket av proangiogenetiska faktorer hos megakaryocyter. Då man ofta ser en ansamling av megakaryocyter i benmärgen hos patienter med PV och KML skulle de kunna ha betydelse för den sjukliga angiogenesen.

Då vi nu inte kunnat påvisa någon specifik markör för patologisk agiogenes i benmärg gick vi vidare med frågan: vilken är mekanismen bakom den abnorma morfologin som ses hos de nybildade kärlen? Man har tidigare påvisat överuttryck av proangiogenetiska faktorer (VEGF och PDGF) i benmärgen vid dessa sjukdomar, men fynden är inte helt samstämmiga. Vi ställde oss frågan om en defekt rekrytering av stödjeceller, pericyter kunde vara en del av svaret.

I delarbete III undersöker vi därför förekomsten av stödjeceller i benmärg hos den sjukdomsentitet som uppvisar den mest uttalade kärlpatologin: myelofibros. Myelofibros är en ovanlig sjukdom där orsaken inte är helt klarlagd. Den tros bero på en mutation i en tidig stamcell och karakteriseras av dålig utmognad av megakaryocyter samt förändringar av förstadier till både vita och röda blodkroppar.

Då pericyter inte tidigare studerats i human benmärg valde vi att använda flera markörer för att identifiera pericyter. Av dessa var det bara antikroppar riktade mot SMA-α (smooth muscle actin) som identifierade en cellpopulation i nära kontakt med endotelceller, vilka omslöt dessa med fingerlika utskott. Vi valde därför att definiera pericyter som SMA-α-positiva celler omgivande CD34-positiva endotelceller som formar ett lumen.

Då vi jämförde förekomst av pericyter hos patienter och kontroller fann vi något överraskande att en större del av kärlen var pericytbeklädda hos patienter med myelofibros jämfört med kontroller. De pericytklädda kärlen visade sig också vara de med den mest uttalat patologiska morfologin med signifikant ökning av både diameter och perimeter.

I detta arbete demonstrerar vi således för första gången förekomst av pericyter i human benmärg. Hos patienter med myelofibros och gravt patologiska kärl ses en ökad förekomst av pericyter, ffa. kring de patologiska kärlen. Hämning av pericytrekrytering skulle alltså kunna ha terapeutisk potential vid myelofibros.

I delarbete IV undersöker vi den molekylära bakgrunden till kärlnybildningen vid myelofibros. Förutom ökad kärlnybildning karakteriseras denna sjukdom av en ansamling av omogna megakaryocyter, i benmärgen. Genom att i möss specifikt manipulera gener som deltar i utmognaden av megakaryocyter kan man få dessa möss att insjukna i tillstånd som liknar den mänskliga sjukdomen.

Möss, som överuttrycker TPO, (trombopoetin) utvecklar snabbt (inom 2-3 månader) myelofibros. Förändringar i benmärg och perifert blod är desamma som vid human myelofibros men sjukdomen utvecklas snabbare. Möss som underuttrycker transkriptionsfaktorn GATA-1, utvecklar också ett tillstånd som är snarlikt människans myelofibros. Till skillnad från möss som överuttrycker TPO utvecklas detta tillstånd långsamt (först i 15:e månaden).

Både hos möss som underuttrycker GATA-1 och hos möss som överuttrycker TPO har vi påvisat en ökad kärlförekomst (MVD) och förekomst av kraftigt formförändrade kärl i benmärgen. Hos båda modellerna kunde vi också påvisa pericytklädda kärl; liknande de fynd vi beskrivit för patienter med myelofibros.

Dessa resultat visar att den abnorma kärlnybildningen vid myelofibros är intimt

kopplat till den störda utmognaden av megakaryocyter vid denna sjukdom.

I min avhandling har jag alltså förgäves sökt efter en markör för nybildade kärl i benmärgen hos patienter med myeloproliferativ sjukdom. Vi kunde visa att nybildade kärl i benmärg skiljer sig från kärl i solida tumörer, men också att det finns proagniogenetiska faktorer uttryckta i megakaryocyter. Dessa i början nedslående resultat sporrade oss att byta infallsvinkel och istället inrikta oss på de formförändrade kärlen vid myelofibros. Vi har där kunnat visa att dessa kärl ingalunda lider brist på pericyter, utan att det tvärtemot verkar ske en överrekrytering. Genom att använda oss av genetiskt manipulerade möss har vi fått belägg för att både den ökade agiogenesen och den ökade förekomsten av pericyter är direkt kopplat till den defekta utmognaden av megakaryocyter som ses vid denna sjukdom.

Det är min förhoppning att dessa små pusselbitar av kunskap ska kunna leda till nya strategier i jakten på specifika terapier.

ABBREVIATIONS

CML	chronic myeloid leukaemia
PV	polycythemia vera
MF	myelofibrosis
EC	endothelial cell
VEGF	vascular endothelial growth factor
NOS	nitric oxide synthase
Ang	angiopoietin
VEGFR	vascular endothelial growth factor receptor
MMP	metalloprotease
SMC	smooth muscle cell
ECM	extra-cellular matrix
PC	pericyte
PDGF	platelet derived growth factor
PDGF-R	platelet derived growth factor receptor
TGF	transforming growth factor
UPA	urokinase plasminogen activator
b-FGF	basic fibroblast growth factor
HIF	hypoxia inducible factor
SMA	smooth muscle actin
HMW-MMA	high molecular weight melanoma antigen
RGS	regulator of G-protein signaling
SAGE	serial analysis of gene expression
TEM	tumor endothelial marker
MVD	microvascular density
HPF	high power field
ECP	endothelial cell proliferation
ALL	acute lymphoid leukaemia
AML	acute myeloid leukaemia
dMRI	dynamic magnetic resonance imaging
PET	positron emission tomography
MM	multiple myeloma
NHL	non-hodgekin lymphoma
CMPD	chronic myeloproliferative disorder
ET	essential thrombocythemia
FISH	fluorescence in situ hybridization
IL	interleukin
IGF	insulin like growth factor
SCF	stem cell factor
TPO	thrombopoietin
WHO	world health organization
vWf	von Willebrand factor
Cox	cyklooxygenase
HRP	horseradish peroxidase
TAF	tumor-derived adhesion factor
WT	wild type
TNF	tumor necrosis factor

REVIEW OF THE LITERATURE AND PRESENT INVESTIGATION

Introduction

The myeloproliferative disorders (chronic myeloid leukemia (CML), polycythemia vera (PV), essential thrombocythemia and myelofibrosis (MF) are chronic bone marrow diseases, which all are characterized by abnormal angiogenesis. Except for in CML, no curative treatment is available and therapy is very much directed towards symptom relief. Of these four conditions MF show the highest degree of neo-vessel formation and is also the one with worst prognosis. The aim of the studies compromising this thesis has been to understand the molecular mechanisms for this pathological angiogenesis, so that in a future perspective, specific therapies directed against the pathological vessels can be developed.

Angiogenesis mechanisms in health and disease

Vasculogenesis and angiogenesis

Blood vessels are the highways, roads and paths by which nutrients, blood and immune cells are delivered to all tissues in our body. The walls of vessels are composed of endothelial cells and mural cells (pericytes, smooth muscle cells), which are embedded in an extra-cellular matrix.

In the embryo the vasculature forms by both vasculogenesis and angiogenesis. Vasculogenesis refers to the de novo forming of a primitive vascular network. It involves proliferation and differentiation of endothelial cells (EC), but also their interaction between mural cells and basement membrane components (Fig 1).

Angiogenesis is the remodeling of an established network of blood vessels, by sprouting or intussusception. Angio-genesis is also seen in the healthy adult in the uterus and ovary during the menstrual cycle and in the placenta during pregnancy [1]. Angiogenesis is an essential part in most diseases, e.g. inflammatory, neoplastic and degenerative.

Vasculogenesis - Formation of immature vasculature

In the embryo vasculogenesis is thought to be initiated from mesoderm by differentiation of hemangioblasts [2]. This process is initiated by vascular endothelial growth factor (VEGF) signaling [3]. The $CD31^+$, $CD34^+$ and VEGFR-2 positive hemangioblasts are thought to differentiate into either endothelial or hematopoietic cells and form the blood islands of the yolk sac, but the exact mechanism is still poorly defined.

In vitro, it has been shown that a subset of mesodermal stem cells (defined by the expression of VEGF-R2 (Flk) and GATA-1) lose their ability to differentiate to endothelial cells, and is restricted to become hematopoietic progenitors [4]. Conversely, mesodermal cells lacking GATA-1 give rise to endothelial cells, but these early endothelial cells can also give rise to hematopoietic progenitors.

The blood islands form a primitive vascular plexus that gives rise to the dorsal aorta, the cardinal vein and the embryonic stem cells of yolk sac arteries and veins [5].

Angiogenesis-the remodeling of the primitive vascular network

Angiogenesis can take two forms: sprouting and intussusception [6]. Sprouting angiogenesis comprises endothelial cell proliferation, proteolytic degradation of extra-cellular matrix and EC maturation to functional capillaries. It is facilitated by hypoxia, which up-regulates a number of molecules, including nitric oxide synthase (NOS), VEGF and angiopoietin (Ang)-2. Through the actions of these molecules the primitive vessels dilates by means of nitric oxide and becomes leaky (VEGF). The basement membrane is degraded by matrix

metalloproteases (MMP2, MMP3 and MMP9). Plasma proteins diffuse through the leaky vessels and serve as a provisional matrix. Endothelial cells migrate through interactions between integrins and the matrix, and proliferate in response to VEGF. Ang-2 facilitates sprout formation in the presence of VEGF and these sprouts anastomose to form vascular loops and networks [5].

In intussusception, EC start proliferating within a vessel, resulting in an enlarged lumen that is split by transcapillary pillars. Intussusception occurs during lung and heart development.

Stabilization of immature vasculature, recruitment of pericytes

These newly formed vessels are stabilized by recruitment of mural cells (pericytes and smooth muscle cells (SMC)), and by generation of extra-cellular matrix (ECM).

Pericytes (PC) are cells of mesenchymal origin that form the mural coat around the vessels. They are recruited through the action of platelet-derived growth factor (PDGF) expressed by endothelial cells and pericytes, by signaling through its receptor PDGFR-β, expressed on pericytes. The importance of PDGF signaling has been shown experimentally by studies of the PDGF knockout mouse, which lack pericytes in certain vessels and exhibit microvascular aneurysms[7]. Vessels are also stabilized through the action of Ang-1 and Ang-2 signaling through their receptors Tie-1 and Tie-2[8]. Ang-1 is thought to stabilize nascent vessels and make them leak resistant, while Ang-2 works as its antagonist in the absence of VEGF. Only in the presence of VEGF, Ang-2 facilitates sprouting.

ECM production is stimulated by TGF-β, a multifunctional cytokine that also induce differentiation of mesenchymal cells to mural cells [9,10]. It is expressed by a number of cell types, including EC and mural cells, and dependent of site and context it can have both pro- and anti-angiogenic functions. Signaling through different receptors (RI, R2 and endoglin) regulates this balance. Endoglin (CD105) signaling is thought to orchestrate the multiple actions of TGF-β, a hypothesis that is supported by the studies of endoglin knockout mice. These mice exhibit normal vasculogenesis, but undergo embryonic lethality because of defective vascular remodeling and SMC differentiation.

Branching and remodeling of vasculature

The final optimal pattern of the vascular network in each organ develops by the action multiple pro- and anti-angiogenic factors. Some of these are also involved in regulating branching of the nervous system (such as ephrins and neuropilins)[11,12]. Other factors derive from the ECM, which serves as a store for various growth factors. Metalloproteases (MMP2, MMP3 and MMP9) and urokinase plasminogen activator (UPA) degrades the ECM and release various proangiogenic growth factors such as VEGF and basic fibroblast growth factor (bFGF), which are sequestered in the matrix after release from producing cells. The MMP:s can also generate anti-agngiogenic molecules, such as angiostatin from plasminogen.

Vessel specialization

The last and least understood step in the maturation of vessels is the process of vessel specialization. It includes arterio-venous and lymphatic determination and EC differentiation to form organ specific capillary structures. There is now evidence that differentiation into arteries and veins starts early, at the level of the hemangioblast, and is guided through signaling of the ephrins. TGF-β-Alk 1 and VEGF-VEGFR2[5] promote arterial differentiation. Endothelial specialization includes fenestrations of EC in endocrine glands, cell-cell junctions of the blood brain barrier and sinusoids of the spleen. How the local or mechanical microenvironment control these processes remains to be determined.

Figure 1

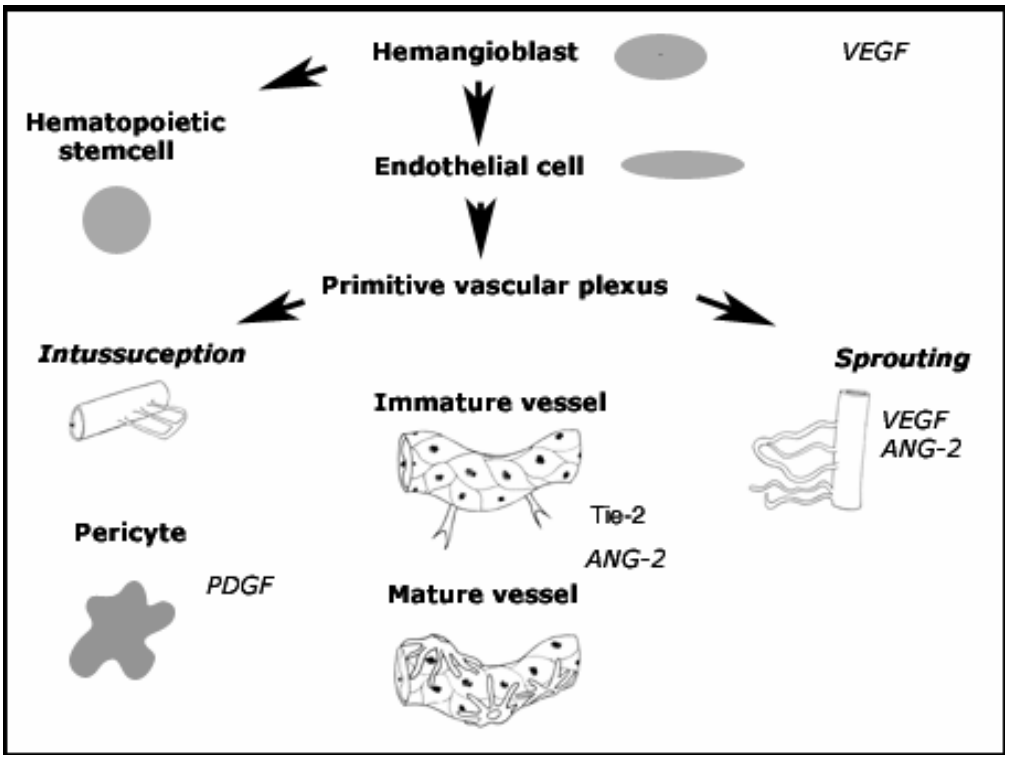

Fig 1. Blood vessel development
The formation of blood vessels starts in the embryo in a process called vasculogenesis. It is initiated from mesoderm by differentiation of hemangioblasts mediated by VEGF signaling. The hemangioblasts have the capacity to differentiate into either endothelial or hematopoietic cells and form the blood islands of the yolk sac. In the embryo, angiogenesis can take two forms: sprouting and intussusception. In sprouting angiogenesis endothelial cells proliferate in response to VEGF and expand after proteolytic degradation of extra-cellular matrix. Ang-2 facilitates sprout formation in the presence of VEGF and these sprouts anastomose to form vascular loops and networks. In intussusception, EC start proliferating within a vessel, resulting in an enlarged lumen that is split by transcapillary pillars. In tumor angiogenesis, endothelial cell progenitors can be recruited from the bone marrow to form new vessels. Tumor cells them cell can also make up part of the new vessel (mosaicism).

The newly formed vessels are stabilized by recruitment of pericytes through the action of PDGF expressed by endothelial cells and pericytes. Vessels are also stabilized through the action of Ang-1 and Ang-2 signaling through their receptors Tie-1 and Tie-2.

Angiogenesis in the adult

Angiogenesis in the adult is a tightly regulated process that only occurs physiologically during the female reproductive cycle. It is also a vital part in many pathological conditions such as cancer, wound healing, diabetic retinopathy, remodeling of tissues secondary to trauma, inflammatory and degenerative diseases, to mention a few.

It follows the same stages as in the embryo and it is assumed that the same molecules are involved, but it has been hard to prove experimentally.

Pathological angiogenesis

Abnormal angiogenesis characterizes several human diseases, but it has been most extensively studied in solid tumors.

A tumor mass needs its own vessel supply to grow larger than 1 mm^3, since cells need to be located within 200-300 mm of blood vessels-the diffusion limit for oxygen. Whether a tumor will continue growing beyond this size depends on whether it can induce the "angiogenic switch" or not, i.e. switch the balance between pro-and anti-angiogenic factors in its microenvironment [13-15]. The angiogenic switch can be triggered by various kinds of metabolic stress (hypoxia, hypoglycemia, low pH), mechanical stress (pressure generated by the growing cell mass) and genetic mutations (activation of oncogenes that control regulation of angiogenic factors). Although all of these events can trigger angiogenesis, hypoxia has been shown to be the major cue for solid tumors. The growing cell mass will consume oxygen, which will result in local hypoxia and induce increased transcription of VEGF-A, the major initiator of angiogenesis. This is mediated by binding of the hypoxia inducible factor (HIF)-1α to a hypoxia response element in the VEGF-A promoter [16,17]. VEGF-R1, Tie-2, NOS are similarly induced by hypoxia. Thus, the balance is changed and angiogenesis initiated.

*Formation of tumor vessel*s

Tumor vessels develop from preexisting vessels in the same way as in the embryo, by sprouting or intussusception, as described earlier. Circulating bone marrow derived endothelial precursors can also contribute to new vessel formation [18], and there is evidence that the tumor cells themselves can make up part of the new vessel wall [19].

Angiogenesis induced by tumors seem to involve the same molecules as in physiological angiogenesis, but due to the dysregulated machinery of the malignant clone, the resulting vessels have an abnormal structure and morphology. They also become chaotically organized and do not follow the hierarchical organization seen in normal vasculature[20]. Typically, vessel diameters are uneven, with dilatations and irregular narrowings. The endothelial cells often show fenestrations and they may not express common endothelial markers such as CD31 or CD34. The molecular mechanisms for this deregulated vessel formation are poorly understood. However, gene targeting and inactivation of Tie-2 [21] PDGF-B [22], α5β1 integrin[23] and endoglin[24,25], has been associated with rather similar gross appearance of vessels.

Tumor vessels may also have a defect maturation, and irregular coverage of pericytes. However, the data in this field is contradictive, with some studies showing lack of pericytes on tumor vessels and some showing abundance [26]. One of the reasons for the controversy might be the difficulty to correctly identify pericytes. These supportive cells are identified by means of their content of smooth muscle actin-α (SMA-α), expression of the receptor for PDGF-B, desmin, high molecular weight melanoma antigen (HMW-MMA), amino-peptidase N or the regulator of G-protein signaling-5 (RGS5) [27]. The proper identification of pericytes is hampered by the fact that the expression of these molecules changes with stage of differentiation and is also tissue specific, and no single marker identifies all pericytes. The physiological role of pericytes is not yet fully understood, but they are thought to be important for vessel stabilization, remodeling and maintenance. There is also evidence that they take part in transport across the blood brain barrier and regulation of permeability [27,28].

Both intravital and immunostainings have shown that tumor-associated pericytes have abnormal morphology and do not form proper contacts with the extra cellular matrix [5].

In summary, tumor vessels differ from the normal vessels in three different ways:

- Tumor cells and endothelial cells up-regulates pro-angiogenic factors and receptors that can be detected within the tumor vasculature.

- Tumor vessels show abnormal morphology and hierarchical organization.
- Tumor vessels may have an abnormal coverage of pericytes and, thus, defective maturation.

Targeting tumor vessels

Targeting tumor vessels could not only have therapeutic implications but also give important information about tumor behavior and prognosis.

In search of new therapies, targeting the tumor vessels rather than the tumor cells themselves has many theoretical advantages: Firstly, endothelial cells are readily exposed to the blood stream, which facilitates drug delivery. Secondly, since each capillary supports hundreds of tumor cells a significant bystander effect could be expected. Finally, endothelial cells are genetically stable which decreases the risk of resistance. However, this area of research has been hampered by the striking phenotypic heterogeneity of tumor vasculature. Each vessel and tumor type seem to have its own specific set of molecules that gets up-regulated, calling for a specific anti-angiogenic therapy for each tumor.

Using nanoparticles coated with peptides from phage libraries, surface expressed nucleolin [29,30] has been suggested to be specific for tumor lymphatic vessels, but the clinical significance remains to be proven.

Using phage display, Ruoslahti *et al* identified peptides that home specifically to tumor endothelium. However these peptides had very low affinity to their target antigens, which made identification challenging [31].

The use of antibody phage display and DNA phage display might overcome some of these problems and has led to the identification of aminopeptidase N as potential tumor specific target [32].

St Croix *et al* performed serial analysis of gene expression (SAGE) on human endothelial cells isolated from normal colonic mucosa and colorectal tumors, and could identify nine tumor endothelial markers that was over-expressed both DNA and RNA level [33]. Four of these tumor endothelial markers (TEM)s are located on the cell surface and thus attractive targets for therapy, namely TEM 1 (endosialin) TEM 5, TEM 7 and TEM 8 [34]. However, more experimental studies are needed to prove the use of these markers therapeutically.

The abnormal morphology and function of tumor vessels can in part explain refractoriness to intravenously delivered cytotoxic drugs. Normalization of the vasculature would, therefore, have the potential to increase the efficacy of both conventional cytotoxic and specifically targeted therapies.

Alternatively, interfering with the EC-pericyte interactions may destabilize tumor vessels and make them more vulnerable for anti-VEGF therapy [35]. However, the great heterogeneity in pericyte coverage of different tumors makes this strategy not applicable to all tumors.

Methods of quantification of angiogenesis

Histological quantification of tumor angiogenesis only started in 1991 when Weidner *et al* identified vessels using an antibody directed against FVIII related antigen[36]. Since then, counting the microvascular density (MVD) has proven to be a powerful prognostic tool in several human solid tumors [37-39].

The method used by Weidner *et al* involved identification of so called hot spots, areas of intense vascularisation. The number of immunohistochemically stained vessels was then counted at high power (x250-x400) and the mean of 3-5 high power fields (HPF)s were presented. Vessels were identified as any endothelial cell or endothelial cell cluster separate from adjacent micro-vessels, thus an identification of a lumen was not a compulsory criteria [40].

This technique has been used in many studies but suffers from considerable

problems with subjectivity when selecting the hot spots, and also in correctly identifying vessels. To overcome this problem a 25 dot Chalky microscope eyepiece graticule that gives a measure of the area covered by vessels (Chalkey counting) was introduced [41]. This method is not only objective, but also rapid and reproducible. To identify endothelial cells a number of antibodies have been used e.g. factor VIII-related antigen, CD31 and CD34. In the second international consensus on the methodology and criteria of evaluation of angiogenesis quantification in solid tumors, CD34 has been recommended as the most specific and reproducible marker [42].

To facilitate assessment of tumor angiogenesis a vascular grade based on the subjective judgment of trained observers also have been used [43]. This method is time saving, and reproducible, but of course utterly subjective, which makes it difficult to use in multi center studies.

An alternative method is to highlight only tumor associated endothelium that is undergoing active neo-vascularisation. A number of antibodies, reported to recognize antigens up-regulated in tumor endothelium has been used e.g. EN7/44, CD105 (endoglin) and endosialin [43]. The number of CD105 positive vessel has been shown to have an association with prognosis in breast cancer[44]. Unfortunately, the wide phenotypic variation of tumor vessels between different tumor types and stages limits this approach [33].

Another way of targeting activated tumor endothelium is to measure endothelial cell proliferation (ECP). Tumor tissue sections are stained with antibodies directed against an endothelial cell marker (CD34) and a marker of proliferating cells (Ki-67). The ratio of tumor to ECP ratio will then reflect the degree of angiogenesis dependent tumor growth [42].

To study the functional status of the tumor vessels and vessel maturity, the amount of pericyte coverage can be measured. By staining for both endothelial cells (using an anti-CD34 antibody) and pericytes (using anti-alpha-smooth muscle actin) the pericyte covering index can be calculated. It has been shown not only to differ widely between different tumors [45], but also between different tissues.

Angiogenesis in hematological malignancies

In hematological malignancies, bone marrow and lymphatic organs harbor the malignant clone that gives rise to disease. In primary bone marrow malignancies the malignant clone develops in a heavily perfused, oxygenated environment and the need for neo-angiogenesis is not as apparent as for solid tumors. However, during the last decade it has been shown that angiogenesis plays an important part in the development of most hematological malignancies, and has also prognostic and therapeutic implications.

Activation of angiogenesis

As in solid tumors, HIF-1α induced up-regulation of VEGF, as well as up-regulation of angiogenic cytokines and down-modulation of anti-angiogenic factors by oncogenes are currently thought to be the basic mechanisms responsible for the angiogenic switch in hematological malignancies [46]. The pro-angiogenic molecules can be produced by the malignant cells themselves, by normal hematopoietic cells, as well as by cells of the bone marrow stroma.

Synthesis of the strong inducers of angiogenesis, VEGF and bFGF, has been demonstrated for leukemia cells, non-Hodgkin's lymphoma (NHL) and myeloma cells [47,48]. VEGF stimulates endothelial cell proliferation, sprouting and release of growth factors in a paracrine loop [49]. An autocrine inhibition of apoptosis and promotion of growth of the malignant clone itself, leads to further expansion of the malignant clone[50,46]. Platelets and megakaryocytes contain VEGF, bFGF and PDGF, which are important in diseases with increased megakaryocytopoesis, such

as the myeloproliferative disorders[51]. Stromal cells, such as osteoclasts can be activated by myeloma cells to produce VEGF and angiopoietin-2 [52,53].

By interference of the malignant clone the balance of pro- and anti-angiogenic signals are not tightly regulated, resulting in a disorganized vasculature similar to that of solid tumors. Lundberg et al described pathological branching in PV and CML. The most striking aberrations were found in MF with highly branched and tortuous vessels[54]. Also in acute lymphoblastic leukemia (ALL) complex, branched vessels were found using a computer-aided three-dimensional reconstruction of bone marrow vasculature [55].

Quantification of bone marrow angiogenesis

Determining the MVD has been shown to be of prognostic value in many solid tumors as well as in hematological malignancies. However, the MVD is the net result of angiogenesis and angioregression, processes that occur simultaneously. It is, thus, a misconception to assume that the MVD is a measure of the angiogenic activity at the time of biopsy and it cannot correctly reflect the effect of anti-angiogenic therapy[56]. Moreover, the functional status (proliferation, maturity) cannot be determined.

Several different antibodies have been used to identify bone marrow endothelium and they have all their advantages and drawbacks. FVIII staining is not very sensitive and will also stain megakaryocytes. *Ulex europeus* also labels some inflammatory and neoplastic cells. Immunohistochemistry using anti CD31 has been considered as the golden standard to measure MVD in the bone marrow [46]. The main disadvantage with this technique is that it will also label megakaryocytes and platelets. A cut slice of a megakaryocyte will, thus, be indistinguishable from a vessel. In disorders with increased megakaryocytopoesis this will be a major disadvantage. CD34 is a very sensitive and robust marker but carries the disadvantage of staining myeloid blasts, making it unsuitable for assessment of MVD in CD34-positive acute myeloid leukemia (AML) [57]. However, due to its reliability, it has been used in the majority of studies dealing with bone marrow angiogenesis [58]. CD105 (endoglin) has been proposed to preferentially recognize newly formed, angiogenic vessels [59,60]. To get a correct picture of ongoing angiogenesis, techniques that quantify the ECP fraction can be used as described above. It has been shown that normal quiescent endothelium with a life span of over 100 days will have an ECP fraction of 0.01% while endothelium of hematological malignancies have an ECP varying between 0 and 7% [61].

Non-invasive assessment of angiogenesis is possible using doppler sonography or contrast-enhanced dynamic magnetic resonance imaging (dMRI) and positron emission tomography (PET) [62]. These techniques do not allow a direct quantification of MVD, but provide information about the functional status of the blood vessels such as permeability.

Measurement of angiogenic molecules in plasma and urine has also been used to provide prognostic information. However, the levels measured in plasma will not always correspond to the expression in the bone marrow compartment and gives no information what type of cells are producing them. Angiogenic molecules can be produced by cells of the peripheral blood, which may give confounding results. Circulating platelets and mononuclear cells are rich sources of VEGF which is released during the coagulation process [63-65]. It is also possible that VEGF is taken up and stored by platelets [66].

Evidence of increased angiogenesis in hematological malignancies

Increased angiogenesis has been shown in virtually all of the hematological malignancies, including MDS [67], AML

[68,69], ALL [55], multiple myeloma (MM) [70,71], non-Hodgkin-lymphoma (NHL)[72].
Increased MVD or elevated levels of angiogenic factors have been proposed as adverse prognostic factors in these disorders.

Myeloproliferative disorders
Increased MVD has also been observed in the chronic myeloproliferative disorders (CMPD) (PV, essential thrombocythemia (ET), CML and MF). MVD has also been shown to be a significant adverse prognostic variable in all the CMPDs [73].

In PV, the increase in MVD is only moderate, but the vessels show pathological branching as mentioned above [54].

In ET, MVD is also only modestly increased, but the plasma levels of VEGF is significantly higher than that of the other CMPDs. VEGF activates endothelial cell exocytosis of Weibel-Palade-bodies, releasing vasoactive substances capable of causing vascular thrombosis and inflammation [74], suggesting a role for VEGF in the thrombotic complications of ET.

In CML, the MVD is highly increased and the vessels also display an aberrant morphology with abnormal branching and varieties in luminal distension similar to those found in solid tumors [54]. Plasma levels and bone marrow expression of VEGF have also been shown to be increased in CML [75].

It has been shown that the bcr-abl-fusion gene, that characterizes the malignant clone in CML, also is present in the endothelial cells of the bone marrow. These data suggest that CML arises from a hemangioblastic progenitor cell, giving rise to malignant blood cells as well as clonal endothelial cells [76,77]. These data also provide evidence for the existence of hemangioblasts in the adult bone marrow, which potentially could contribute to post-natal vasculogenesis.

Among the myeloproliferative disorders, the most pronounced increase of angiogenesis is seen in MF [78]. In MF there is clonal proliferation and deficient maturation of megakaryocytes, a rich store of cytokines and pro-angiogenic factors. When released, they cause an intensive stromal reaction and increase in bone marrow vascularity [79,80]. Several cytokines have been implicated in this process.

TGF-β was shown to be elevated in patients peripheral blood mononuclear cells, and also localized to megakaryocytes [81]. In a later study, no over-expression of TGFβ was seen in the bone marrow, but its receptor TGF-βR1, was shown to be over-expressed in small bone marrow vessels [82].

bFGF has been detected in circulating megakaryocytes and platelets of MF patients [83], and it has been shown to be over-expressed in patient bone marrow megakaryocytes [82].

Increased serum-levels of *VEGF* and increased expression in the bone marrow has been demonstrated for patients with MF [54,84,85]. These data are now being questioned in a larger study by Teferi et al who measured bone marrow expression of VEGF in 66 patients with MF, and could find no significant increase when compared to controls [86]. This discrepancy might in part be explained by differences in diagnostic criteria in the different investigations, a matter that will be discussed below.

PDGF, a cytokine constitutively expressed by megakaryocytes, is responsible for the recruitment of pericytes to newly formed vessels and induces VEGF expression in endothelial cells [87]. It has been suggested to contribute to the increased angiogenesis in MF, but the evidence are so far only indirect [88].

The increase in MVD correlates with cellularity, megakaryocyte clumping, spleen size and over all survival in MF [78].

Myelofibrosis-pathogenesis and diagnostic considerations
Myelofibrosis is a clonal stem cell disease with tri-lineage proliferation and a secondary intense stromal reaction

including collagen fibrosis, osteosclerosis and angiogenesis [89]. Symptoms include progressive anemia and massive splenomegaly and the blood smear typically shows teardrop shaped cells, nucleated red blood cells and myeloid precursors. Average life expectancy is estimated at 5-7 years and cause of death includes leukemic transformation in 10%-20% of patients the first 10 years. MF can present de novo and is then termed idiopathic myeloid metaplasia, or develop in patients with PV (post-polycythemic myeloid metaplasia) or in patients with ET (post-thrombocythemic myeloid metaplasia) [89]. The current treatment is directed against palliation, relieving symptoms of anemia and splenomegaly. Allogenic bone marrow transplantation is the only treatment with a potential cure, but since the majority of patients are above 60 years at onset of disease, very few qualify for this treatment.

Pathogenesis

Since MF affects both myeloid and lymphoid lineages it is generally believed that the disease is clonal and arise from an early stem cell [90]. However, the disease-causing mutation is still unknown. No specific cytogenetic abnormality has yet been liked to the disease, neither by chromosome analysis or by fluorescence in situ hybridization (FISH) [91,92]. However, gene expression studies have been able to identify disease specific down-regulation of RARβ2 and BCLI and up-regulation of HMGA2, FKB51, GATA2, and JUNB, in MF derived CD34 cells. However, these genes are not located on the same chromosome and the underlying mechanism causing the dysregulation of these genes is still unknown [93-95].

It has been shown that in the early hypercellular phase of the disease, there is an increase in $CD34^+$ cells in the bone marrow. In the later fibrosclerotic phase, the numbers decrease, but a corresponding elevation is seen in the spleen and they are also detected in the peripheral blood [96]. These circulating $CD34^+$ cells exhibit the markers of primitive progenitors, being $CD38^{low}$ and Thy-1^+ and expressing the c-kit multi-lineage receptor [97] and are thus believed to be part of the malignant clone.

The stromal reaction is thought to be reactive and mediated by cytokines derived from clonal megakaryocytes and moncytes[89]. Over-expression of TGF-β1 has been specifically linked to the development of fibrosis[98], and osteo-protegerin to osteosclerosis[99].

As mentioned before, there is also over-expression of PDGF, bFGF and VEGF, thought to contribute to the characteristic increase in angiogenesis. In the spleen, the presence of progenitors with a hemangioblastic phenotype ($CD34^{+,}$ $AC133^+$, $VEGFR2^+$) has been demonstrated[100]. These progenitors have a high potential for endothelial proliferation and could contribute to post natal vasculogenesis in myelofibrosis [101].

Lately, much research has focused on the importance of JAK kinases for the development of myeloproliferative disorders. JAK kinases regulate the down-stream signaling of type I cytokine receptors, including erythropoietin (EPO), stem cell factor (SCF), GM-CSF, interleukin 3 (IL-3), thrombopoietin (TPO) and insulin growth factor-1 (IGF-1). Hematopoietic stem cells in CMPD have been shown to be hypersensitive to these cytokines, and mutations in their receptors have been thought to be the cause. However, except for the situation in familial erythrocytosis (shown to be caused by a mutation in the EPO-receptor) [102], no such mutations have been specifically linked to any of the myeloproliferative disorders [103]. These cytokines all use the JAK2 kinase, and by a cumbersome sequencing effort, Green et al a identified a somatic missense mutation in the JAK2 gene in 97% of patients with PV, 57% of patients with ET and 50% of those with MF, but in none of their 90 normal control samples [104]. Likewise, a group headed by William Vainchenker found that 89% of

patients with PV, 43% of those with ET and 43% of patients with MF bear the JAK2 mutation, but in none of their controls or in patients with secondary erythrocytosis [105].

Diagnosis

In the absence of a biological marker for MF the diagnosis of MF has been based on the presence of typical symptoms and signs (bone marrow fibrosis, splenomegaly, anemia, poikilocytosis) and the exclusion of other hematological disorders, presenting with a similar picture. Diagnostic criteria have been the matter of intense debate and two groups have independently proposed two different ways of diagnosing and staging the disease.

In 1998 the Italian consensus conference published their diagnostic criteria based on the notion that no casual biological hypothesis currently identifies the principal parameters for the diagnostic definition and that only a consensual approach could be possible. An expert panel selected 2 necessary criteria (presence of diffuse bone marrow fibrosis and absence of Philadelphia chromosome or CRAB re-arrangement in peripheral blood cells) and six optional (splenomegaly, presence of anisopoikilocytosis with tear-drop erythrocytes, presence of immature myeloid cells or erythroblasts, presence of clusters and anomalous megakaryocytes in the bone marrow or myeloid metaplasia [106]. To allow for a diagnosis of MF the two necessary criteria plus two optional must be fulfilled when splenomegaly is present. If splenomegaly is absent four of the optional criteria must be fulfilled. It is noteworthy that anemia is not present as a criteria, although thought of as a typical sign. The reason is to not exclude myelofibrosis developing from a setting of PV, where the red cell blood count could be persistently elevated.

In 1999, Thiele and coworkers published an approach based on the hypothesis of the presence of a prefibrotic phase/type of the disease, the Cologne criteria [107], thus excluding bone marrow fibrosis as a necessary criteria for MF. Instead the presence of splenomegaly, leuko-erythroblastic picture of peripheral blood, anemia, thrombocytosis and clustering of megakaryocytes were used to define the diagnosis and progression of the disease. The World Health Organization (WHO) has also accepted these criteria.

Mouse models of myelofibrosis

To gain further insights in the molecular mechanisms underlying MF, research has focused on the abnormal proliferation and maturation of megakaryocytes. Mega-karyocytic maturation is regulated extrinsically by TPO [108] and intrinsically by expression of transcription factors, of which GATA-1 has been shown to be one of the most important ones [109,110]. There are now two models where mice have been genetically manipulated to either over-express TPO, or have a low expression of GATA-1. Both these models develop a disease sharing many of the characteristic features of the human condition, but with some differences.

In the model initially described by Yan et al [111], mice have been given bone marrow grafts of cells infected with a retrovirus carrying TPO complementary DNA. These mice develop rapidly (within 2-3 months) a myeloproliferative disorder with a prominent proliferation of megakaryocytes in the bone marrow, leukocytosis, thrombocytosis, extramedullary hemato-poiesis, splenic- and medullary fibrosis and sclerosis and die soon thereafter [112]. No over-expression of TPO has been shown in the bone marrow of patients with MF [113], but megakaryocytes from both the animal model and from patients display decreased surface expression of the TPO receptor Mpl, which might result in decreased clearance of TPO and subsequent ongoing stimulation of other cells. This finding is, however, not restricted to MF but applies to all the CMPD:s, excluding it as a disease specific molecular marker [114].

The pivotal role of GATA-1 in erythroid differentiation is demonstrated by the fact that GATA-1 knockout mouse die in utero by severe anemia. However, mice with reduced expression of GATA-1, (GATA-1^{low}) survives, but are born anemic and thrombocytopenic [115,116]. They display a block in differentiation in maturation of megakaryocytes into proplatelets that results in the accumulation of megakaryocytes in the bone marrow and spleen. These mice could thus be considered as a model to study the effects of a chronic increase of megakaryocytes in the absence of alterations in the TPO/Mpl signaling pathways [117]. These mice will, at 15 months of age, develop a MF-like disease with anemia, teardrop poikilocytosis, myeloid progenitor cells in the blood, collagen fibers in the spleen and marrow, and hematopoietic foci in the liver. The slow onset of the disease correlates better to the clinical course of human MF. There is no evidence of under-expression of GATA-1 in human MF [118], but mutations of GATA-1 has been found in dyserythropoietic anemia, X-linked thrombocythemia and thalassemia [119-121].

Thus, neither of these models has relieved the exact molecular mechanism underlying human MF; however, they can give further insight in each of the phenotypic hallmarks of the disease, and possible new ways of targeting them, i.e. the TPO over-expressing mice have been vital for understanding the role of TGF-β1 in MF. Lethally irradiated hosts engrafted with $TGF^{-/-}$ hematopoietic stem cells does not develop reticulin fibrosis as their wild-type counter parts do, demonstrating that TGF-β1 is essential for the promotion of myelofibrosis.

Aims of the present work

The aim of this study has been to investigate the molecular mechanisms underlying the pathological angiogeneis in the bone marrow of patients with myeloproliferative disorders.

Specific aims of the study

My strategies have been to:

-Identify specific markers for newly formed vessels in the bone marrow
-Characterize the expression of pro-angiogenic factors in megakaryocytes in patients with myeloproliferative disorders
-Investigate if abnormal pericyte coverage is part of the pathological angiogenesis seen in myelofibrosis
-Investigate if abnormal angiogenesis is present in the bone marrow of genetically manipulated mice (TPO over-expressing or GATA-1^{low}) displaying a phenotype closely resembling that of human myelofibrosis.

Materials and Methods

In this section materials and methods will be briefly summarized. Detailed descriptions are found in the "Materials and Methods" section of each paper. The ethical committee at Huddinge University Hospital approved all studies including patient material.

Patients (Paper I-III)

Patients with PV, CML and MF that were diagnosed and followed at Stockholm Söder hospital or Huddinge University Hospital were identified. Bone marrow samples taken as part of a diagnostic procedure were collected. The control material consisted of patients evaluated for anemia or leukocytosis, where no

abnormalities could be found either at time of investigation or at follow-up.

Animals (Paper IV)
Bone marrow and spleen biopsies from genetically manipulated mice were kindly provided by Professor Alessandro Vannuchi (University of Florence, Florence, Italy) and Dr William Vainchenker (IFR 54-INSERM U 362, Institut Gustave Roussy, Villejuif, France). Mice were bred and kept in animal facilities approved of by the ethical committee of their respective departments.

Immunohistochemistry (Papers I-IV)
Immunostainings of CD34, von Willebrand factor (vWf), cyklooxygenase 2 (Cox-2), endoglin, Tie-2, angiomodulin and glycodelin are described in paper I, immunostainings of VEGF in paper III. We used established techniques, decorating positive cells with fast red or DAB. Double stainings with Tie-2 and CD34 (paper I) were accomplished by performing the protocols sequentially. Sections were analyzed under a light microscope.

Immunostainings of mouse CD34 and CD31 (paper IV) were performed using monoclonal rat anti-mouse CD34 and CD31 as primary antibodies and a mouse adsorbed biotinylated rabbit anti-rat antibody as secondary. For PDGFR-β, a horseradish peroxidase (HRP)-conjugated mouse anti PDGFR-β antibody was used, and thus no secondary antibody.

Immunofluorescence (Papers III and IV)
To visualize pericytes and co-localization to endothelial cells, immunofluorescence stainings were performed and sections analyzed by fluorescence microscopy. For human material primary antibodies were: for CD34, a mouse monoclonal IgG1; for SMA-α, a FITC conjugated anti-mouse IgG2a; for desmin a monoclonal mouse anti-IgG1, and for PDGFR-β a rabbit polyclonal. Secondary antibodies were: Alexa 568 (red colour) and Alexa 488 (green color). For double stainings with CD34 and SMA-α the two primary antibodies were applied simultaneously, and the two secondary antibodies as well.

Double stainings of mouse sections for CD34 and SMA-α were performed by first staining for CD34 and visualize with fast red. This substrate is visible both in ordinary and fluorescent light. Then, a FITC-conjugated anti SMA-α antibody was added followed by Alexa 488.

To reduce background auto-fluorescence in bone marrow samples, pretreatment with ammonium-ethanol and sudan black were used.

To visualize the 3-dimensional orientation of pericytes to endothelial cells confocal microscopy (Leica Q5501) was used.

Microvascular density (Papers I,III,IV)
In papers I and IV, MVD was analyzed by staining endothelial cells immunohistochemically for CD34 or vWf, as described above. Counting was performed by two separate investigators in a blinded fashion. The five areas with most dense vascularisation (hot spots) were selected, and the mean number of vessels per HPF was determined. In paper I, 200x amplification was used, but in paper IV, 400x had to be used due to the smaller size of vessels in the mouse. In paper III, MVD was determined by using confocal microscopy and vessels were counted in 63x HPFs. To obtain a representative number of vessels, 5 HPFs were counted.

Morphometric analyses (paper III)
To determine perimeters of vessels we used the image analysis system Leica Q5501W with color video camera DM RXA (Wetzlar, Germany) together with a software system for measurements of blood vessel characteristics, developed with the Leica Qwin Image Analysis. Ten randomly selected and crosscut vessels per slide, with a clearly visualized lumen, were captured with a 63x oil immersion objective and the perimeter was assessed by manually tracing the inner (luminal) CD34 staining.

3-dimensional imaging (paper III)

To perform confocal microscopy and 3-dimentional reconstructions of vessels, paraffin embedded tissue was microtomed into 30-50 µm thick slabs. Sections were stained using a standard protocol for immunofluorescence and primary anti-bodies were mouse anti-CD34 and mouse anti- SMA-α. Goat anti-mouse IgG1 Alexa 546 (red color) and goat anti-mouse IgG2a Alexa 488 (green colour) (Molecular Probes, Eugene, OR) were used for detection.

Slides were analyzed using Leica TCS NT confocal laser scanning microscope fitted with air-cooled argon and krypton lasers adjusted for optimal serial section acquisition. Serial optical sections, 0.5 µm thick, were recorded beginning at the top surface of the prepared specimen. Stacks of serial sections were then rendered in three dimensions using VoxelView 2.5.1 software to examine the 3-dimensional architecture of the endothelial wall constituents. Vessels, selected for 3-D analysis study were identified by the presence of CD34 positive cells. Projections sequences were constructed from these data without filtering or segmentation.

Statistical analyses

Analyses were performed with the Statistica TM software package using Student´s t-test, χ^2-test and ANOVA. Results are given as mean and SD values. P values <0.05 were considered as significant.

Results and Discussion

Characterization of vessels and expression angiogenic factors in the bone marrow of patients with myelo-proliferative disorders (Papers I and II)

The aim of this study was to investigate whether pro-angiogenic molecules, over-expressed on vessels of solid tumors also were over-expressed in the bone marrow in patients with myeloproliferative disorders.

At the time of the study there was still some controversy on which marker that was best fitted to label bone marrow endothelium. Thus, we started out by determining MVD with two different markers, vWf and CD34. Both markers labeled bone marrow vessels, but MVD measured by vWf was almost twice that of CD34. This raised a question of specificity, since vWf also labels megakaryocytes, which are accumulated in the bone marrow in patients with myeloproliferative disorders and a crosscut megakaryocyte can hardly be distinguished from a vessel. We chose to use CD34 as a marker for endothelial cells and for the determination of MVD. We could repeat previously obtained results showing increase in MVD in patients with CML and PV (5.4±3.6 and 4.2±5.1 vessels/HPF, respectively) as compared to controls (2.6±1.2).

Next, immunostainings for Tie-2, Cox-2, endoglin, glycodelin and angiomodulin was performed. Of these, only Tie-2 stained bone marrow endothelium. Since the other antibodies stained other cells in the bone marrow, the possibility of a false negative result was excluded.

We, then, tried to see if Tie-2 was over-expressed on newly formed pathological vessels in patient bone marrows. When performing double stainings of CD34 and Tie-2 it was found that most vessels expressed both CD34 and Tie-2, but there was a fraction of cells only expressing CD34 and one only expressing Tie-2. There was a tendency that PV patients had a larger fraction of vessels expressing only Tie-2, but it did not reach statistical significance.

Tie-2 is a receptor for angiopoietin-I and II, known to be important in vasculo-genesis and maturation of vessels. It is expressed in endothelial and hematopoietic cells, is believed to play a role in both angiogenesis and hematopoiesis during

development [122]. It has been shown to be over-expressed in endothelium of solid tumors. Tie-2 signaling mediates survival for hematopoietic progenitors and AML blasts [123,124].

In paper I, we demonstrate for the first time the expression of Tie-2 on bone marrow endothelium and in paper II, the expression of Tie-2 in megakaryocytes. However, we could not show any disease-specific expression in either cell type, making it ineligible as a marker for pathological angiogenesis in these disorders.

Cox-2 is an inflammation-associated enzyme involved in the pathogenesis of many solid tumors, but little is known about its presence and role in hematological neoplasms. It converts arachidonic acid to prostaglandins, which induce expression of angiogenic factors, including VEGF, b-FGF, TGF-β and IL-6. Recently, it was shown to be over-expressed in the bone marrow of multiple myeloma patients. In contrast to the situation in solid tumors, there was no over-expression in endothelial cells, but only in the malignant plasma cells [125]. It has also been shown to be over-expressed in the malignant promyelocytes of CML [126]. In our material Cox-2 also stained megakaryocytes (paper II), which are abundant in these disorders. Thus, Cox-2 might possibly have a role in driving the pathological angiogenesis in myelo-proliferative disorders, but it could not be used as a marker for newly formed pathological vessels.

Endoglin (CD105) is the TGF-β receptor III present on endothelial cells, syncytiotrophoblasts, macrophages, connective tissue stromal cells and mesenchymal stem cells. Endoglin on endothelial cells potentially plays a role in TGF-β signaling in mediating its proangiogenic function [127]. It has been shown to be over-expressed in tumors of mesenchymal origin. In our hands, it did not stain any bone marrow vessels, neither in patients nor in controls (paper I). However, there are now two reports from the same group on CD105 staining of bone marrow vessels in multiple myeloma and myelofibrosis [128,129]. This antibody recognized more vessels than CD34. Since the CD105 staining vessels were small and slit-like the authors draw the conclusion that CD105 recognizes newly formed vessels. No other analysis on proliferation or age was performed. This discrepancy with our results might be due to a difference in antibody specificity. The antibody used in paper I was designed to recognize mesenchymal stem cells who also express CD105, and might have a different affinity to endothelial CD105.

Glycodelin, a glycoprotein secreted by endometrial glands and over-expressed in tumors originating from the female reproductive tract [130] was not expressed by endothelial cells in the bone marrow, but on the contrast in megakaryocytes (paper II). However, its role in promoting angiogenesis remains unclear.

Angiomodulin or tumor-derived adhesion factor (TAF) has been shown be specifically accumulated in small blood vessels and capillaries within and adjacent to tumor nests, but not in those of normal tissues [131]. Tumor blood vessel-specific staining of TAF has been observed in various human cancers, such as esophagus, brain, lung, and stomach cancers. In our studies no expression of TAF was seen in bone marrow vessels neither in patients nor in controls.

These studies demonstrate that angiogenic factors, over-expressed in solid tumors, are not over-expressed on bone marrow endothelium in patients with CMPD. These vessels have thus, their own phenotype and techniques such as phage display and SAGE could be used to further characterize them.

We can also show the expression of angiogenic factors (Cox-2, Tie-2 and glycodelin) in megakaryocytes, which might be of importance for the pathogenesis of these disorders, since they

are characterized by megakaryocyte accumulation in the bone marrow.

Patients with myelofibrosis have an increased amount of pericytes in the bone marrow (Paper III)

In CMPD and especially MF, there is not only an increased microvascular density, but also aberrant vessel morphology. The aim of this study was to study the mechanisms underlying the formation of these pathological vessels. Vessels with similar aberrations have been described in experimental models where endothelial cells have been exposed to high concentrations of VEGF, but they also resemble solid tumor vessels, which have abnormal pericyte coverage. Thus, we wanted to investigate whether over-expression of VEGF and/or abnormal pericyte coverage could be part of the pathological process in myelofibrosis.

Identification of pericytes

Since staining of pericytes in the bone marrow has not been described before, we tried a panel of different markers, known to be able to identify pericytes in other tissues: SMA-α, desmin and PDGFR-β. Of these, only SMA-α stained perivascular cells in both patients and controls. PDGFR-β stained a few perivascular cells in patients, but not in controls. Desmin stained only a few scattered cells in the bone marrow, with no obvious relation to endothelial cells. Since others have described that SMA-α staining identifies PC in most other tissues, whereas desmin and PDGFR-β stainings may be more variable, tissue and vessel specific, we used SMA-α as the pericyte marker in this study.

Vessels in myelofibrosis have an increased perimeter and the number of pericyte coated vessels is increased

When analyzing vessel morphology and pericyte coverage we could show that not only was there an increased MVD in patients as compared to controls (23±9 vs. 4±3 vessels/HPF, respectively, $p<0.001$), but vessels in MF bone marrow had also an increased perimeter (89±61 μm in MF vs. 19±9 μm for vessels in control marrows, $p<0.001$). In figure 2, paper III, it is shown that it was possible to define a maximum perimeter (<100 μm) for normal vessels. Vessels with larger perimeter occurred only in patients with myelofibrosis. In fact, 17.2 % of all MF vessels were larger. We could also show that the number of pericyte-coated vessels was increased in MF bone marrows. In MF patients, 99±3% of vessels were found to be pericyte-coated, compared with only 51±20% in controls ($p<0.001$). Interestingly, it was primarily the large pathological vessels that were pericyte coated (Fig. 3, paper III).

In this study we also find support for the hypothesis that the aberrant morphology of myelofibrosis vessels could be due to an over-expression of VEGF by bone marrow progenitors and megakaryocytes (Fig.1B, paper III). Over-expression of VEGF in the bone marrow have been shown previously by other groups [54], but in two studies of myelofibrosis, no difference in VEGF expression was found [82,86]. Thus, there is no consistent evidence that over- or aberrant expression of VEGF-A contributes to the abnormal vasculature in these patients.

This prompted us to investigate if aberrant pericyte recruitment could be part of the pathological angiogenesis. Pericyte coverage is regarded as a sign of vessel maturity and, quite opposite to what we had expected, we found increased pericyte coverage of the abnormal vessels in myelofibrosis patients. This finding suggests that the abnormal vessels is not newly formed, but have reached a certain stage of maturity. The increased pericyte coverage of the pathological vessels might be secondary to an increased recruitment, depending on secretion of a chemotactic molecule that still is not defined. Current hypothesis suggests that PDGF-B, secreted from endothelial cells or megakaryocytes, is such a factor. There is evidence that

many of the reactive changes seen in myelofibrosis are caused by a block in megakaryocyte differentiation and an overproduction of megakaryocyte growth factors in the bone marrow, of which PDGF-B is one.

Evidence of increased angiogenesis and aberrant vessel morphology in two mouse models of myelofibrosis (paper IV)

To gain further insight in the molecular mechanisms underlying the pathological angiogenesis in myelofibrosis we studied two mice models of myelofibrosis: mice over-expressing TPO and mice with a low expression of the transcription factor GATA-1 (GATA-1^{low}). The aim of this study was to document whether these mice also show increased angiogenesis, aberrant vessel morphology and increased re-cruitment of pericytes.

MVD is increased the bone marrow of GATA-1^{low} -and TPO over-expressing mice

Bone marrow and spleen endothelial cells were stained with antibodies directed against CD31 and CD34. Both antibodies stained vessels in GATA-1^{low} mice, but only CD34 stained vessels in the TPO over-expressing mice. Whether this was due to differences in fixation of the material, or a true lack of expression of CD31, remains to be elucidated. For this reason immunostaining against CD34 was used for the MVD analysis.

In GATA-1^{low} mice we found an increased MVD, both in the bone marrow (11±2.4 vessels /HPF compared to 3.0±0.9 in wild type (WT) controls; $p<0.001$) and in the spleen (20±6 vessels/HPF compared to 5,1±1.6/HPF in WT, $p<0.001$). As also seen in human myelofibrosis, the GATA-1^{low} bone marrow vessels were dilated, tortuous, with changing calibers (Fig. 2, paper IV).

In TPO over-expressing mice MVD was also significantly higher: 6.8±2.2 vessels/HPF compared to 1.9±2.2 vessels/HPF and vessels were strikingly aberrant in morphology.

Absence of TGF-β1 does not restore abnormal angiogenesis in TPO over-expressing mice

Lethally irradiated wild-type hosts were engrafted with bone marrow stem cells from homozygous TGF-β1 null (TGF-$\beta1^{-/-}$) and wildtype (WT) littermates, infected with a retrovirus encoding the murine TPO protein. The mice with no expression of TGF-β1 in the bone marrow, but over-expression of TPO, develop no bone marrow or spleen fibrosis, but all other hallmarks of a myeloproliferative disorder[98].

However, they did show signs of pathological angiogenesis: MVD in mice not expressing TGF-β1 were significantly increased compared to WT mice and even higher than that of TPO over-expressing mice with normal TGF-β1 (7.0±1.4 vessels/HPF vs 9.1±2.8, $p=0.03$) (Fig.1, paper IV). The vessels showed the same, or even worse morphological aberrations (Fig. 4, paper IV).

Abnormal vessels in GATA^{low} and TPO over-expressing mice are covered with pericytes

A panel of antibodies was used to identify pericytes in mouse bone marrow: SMA-α, PDGFR-β and desmin. Of these, only SMA-α stained perivascular cells. We thus used SMA-α to study pericytes in the genetically modified mice. GATA-1^{low} mice had a higher total number of vessels in the bone marrow and also a higher number of pericyte coated vessels, but the relative numbers were comparable. The same was true for TPO over-expressing mice, both in the presence and absence of TGFβ.

Both GATA-1 and TPO regulates megakaryocyte and erythroid differentiation. Both animal models described above show abnormal

megakaryocyte maturation and accumulation of megakaryocytes in the bone marrow. In this study we show that both models have abnormal vessel morphology and high recruitment of pericytes. These findings suggest that the abnormal angiogenesis seen in myelofibrosis is closely linked to the abnormal megakaryocyte differentiation and possibly to angiogenic factors (i.e. VEGF, PDGF and TGF-β1) produced by these cells.

General Discussion

The overall aim of this study has been to investigate the molecular mechanisms regulating the pathological angiogenesis in myeloproliferative disorders.

In the first part of the study we tried to find specific markers for newly formed vessels in the bone marrow. There are many alternative strategies to deal with this problem and, as discussed in the introduction part, it has been extensively studied in solid tumors. We chose to use some of the experiences made this field. Hence, by studying the literature we found a panel of factors known to be over-expressed in solid tumors (Cox-2, Tie-2, endoglin, glycodelin and angiomodulin). Using immunohistochemistry, we investigated if they were also over-expressed in the bone marrow of patients with PV and CML, but one of the factors turned out to be so.

In retrospect, our strategy might be criticized at many levels: For exemple, these were not the only factors known to be over-expressed on tumor endothelium; as mentioned in the introduction part of this thesis, several other factors i.e. nucleolin, aminopeptidase N and endosialin have been suggested to be specific markers for neoangiogenesis. However, using immunohistochemistry we were somewhat tied by the limitations of this technique. In this study we used paraffin embedded material fixed in special media (Stieves solution), taken as part of a diagnostic procedure. We could thus only investigate the few factors where there were antibodies available, which could recognize their epitopes in fixed, paraffin embedded material. Moreover, when using bone barrow biopsies a step of de-calcification must be used, a rough treatment known to destroy many antibody epitopes. Since none of these antibodies had been used on bone marrow biopsies at the time of the study, we were also hampered by the lack of positive controls. We did use other tissues (lymphoid and muscle) but since these tissues were not decalcified they were no true controls. Thus, if an antibody could stain lymphoid tissue but not bone marrow, we had to draw the conclusion that it was the decalcification step that was the culprit, and this antibody had to be abandoned. On the contrary, if an antibody did stain cells in the bone marrow but not endothelial cells, we used this as an internal positive control, drawing the conclusion that a negative result was not due to technical problems, but represented a true lack of expression. As discussed before in the case of endoglin, we probably overlooked the possibility that the same antigen could have different affinities for the antibody, when expressed by different cell populations.

It has since become increasingly clear that the struggle to find one specific marker that will recognize all neo-angiogenic vessels might be fruitless. Tumors are inherently heterogeneous and each tumor type and tumor stage will have its own vessel phenotype. We could not find any over-expression of Cox-2, Tie-2, endoglin, glycodelin or angiomodulin in the bone marrow of patients with PV or CML. In the case of endoglin this might be due to a low affinity for the antibody used, but for the others a true negative result must be suspected, since no other group to this date has been able to show over-expression on bone marrow endothelium. Thus, we can conclude the bone marrow vessels in PV and CML have a different phenotype from

that of vessels in solid tumors. To be able to specifically target these vessels other techniques have to be used.

In paper II we show that three of the factors tested for over-expression on bone marrow endothelial cells (Cox-2, Tie-2 and glycodelin) also are expressed in mega-karyocytes, both in patients with PV and CML and in healthy controls. Staining intensity and percentage of mega-karyocytes stained in each section were approximately the same in patients and controls, but since megakaryocytes are accumulated in the bone marrow of these diseases, they might be important for the development of the pathological angiogenesis.

Moreover, immunohistochemistry is not sensitive enough to detect minor differences in expression. RT-PCR on purified megakaryocytes or northern blotting could be used instead.

In both PV and CML the malignant clone is thought to originate from an early stem cell with capacity to differentiate into myeloid, erythroid and megakaryocytic lineages. In the case of CML it has been shown that the disease specific gene rearrangement (bcr-abl) is also found in endothelial cells. The finding that three factors, normally expressed on endothelial cells and with important roles in angiogenesis, are also expressed in megakaryocytes supports the hypothesis that megakaryocytes take an active part in bone marrow angiogenesis, both in health and disease. Using more sensitive techniques, it might be possible to define whether the immature megakaryocytes characterizing both PV and CML also have an aberrant expression of these factors.

In the third part of the study (paper III) we focused on the aberrant vessel morphology seen in the bone marrow of all the myeloproliferative disorders, but especially so in myelofibrosis. Hypothesizing that not only over-expression of proangiogenic factors (VEGF, bFGF) might be responsible for the morphological changes, we investigated if an abnormal pericyte coverage or morphology could also be part of the pathological process. Pericytes were shown to cover the majority of vessels in patients with myelofibrosis, but only half of the vessels in healthy controls. We used confocal microscopy and three-dimensional imaging, and with these techniques no gross morphological changes could be seen in patient pericytes.

Pericytes are cells of mesenchymal origin and are thought to be important for vessel stabilization and maturation. In the developing vessel they are recruited by the cytokine PDGF, secreted by endothelial cells themselves, but also by megakaryocytes. Signaling through the PDGFR-β is vital for this process as demonstrated by the fact that both the PDGF- and the PDGFR-β knockout mouse lack pericytes[7]. The finding that the number of pericyte-coated vessels is increased in myelofibrosis suggests that these cells also might be part of the pathological process, which might have therapeutical implications.

In myelofibrosis no curative treatment is currently available except bone marrow transplantation. Standard therapy today is largely palliative due to the high mean age of patients. Alternative strategies are under active development, many of which have focused on the abnormal angiogenesis seen in the disease. Of these, thalidomide is the most extensively studied drug. It is thought to exert its angiostatic effect through modulation of cytokines, particularly tumor necrosis factor-α (TNF-α). In 5 small phase II studies positive effects on reducing anemia, thrombocytopenia and splenomegaly was reported [132-135]. However, the doses needed caused adverse effects that led to a drop out rate greater than 50% in three months. In one phase II study by Merup et al no significant clinical effects was seen and 34% of the patients discontinued the treatment because of the side effects [136].

However, combining low-dose thalidomide with prednisone produced a better tolerated and more effective dose in a study by Mesa et al [137].

Imatinib mesylate (STI571, Gleevec™) is a selective tyrosine kinase inhibitor with in vitro activity against c-abl and bcr-abl and can induce stable remission in CML, where it is now considered as fist line therapy. Imatinib has also activity against c-kit (CD117), which is highly expressed on CD34+ cells of patients with myelofibrosis, and against PDGFR. The currently available studies are small and the not well-established enrollment criteria used have created highly variable results, which have hampered interpretation of the results. In a study by Hasselbalch et al, a beneficial effect of Imatinib was seen in three of nine patients and the treatment was shown to be safe in combination with hydroxyurea and interferon-α [138].

There are also more experimental treatments aimed at more specifically target the pathological angiogenesis. SU5416 is a small synthetic tyrosine kinase inhibitor of VEGF-R2. It inhibits VEGF-dependent endothelial cell proliferation in in vitro and in animal models. In addition it also inhibits c-kit and the fms-related tyrosine kinase Flk2 (FLT3) receptor. It might therefore target both angiogenesis and blast cell proliferation in myelofibrosis. In a phase II multi center study of patients with CMPD, 3 patients with MF were enrolled of which one had a significant clinical response, the other two remained stable throughout the study [139].

Therapies directed against pericytes are now also being developed. Interfering with PDGF-signaling has been shown to disrupt already established pericyte-endothelial contacts and causes vessel destabilization [140]. SU6668 is a small synthetic tyrosine kinase inhibitor, which potently inhibits PDGF-R signaling, but also signaling through VEGF-R2 and FGF-R1. In an experimental tumor model it was shown that the combined treatment with SU6668 and SU5416 caused endothelial cell apoptosis, blood vessel destabilization and regression and finally tissue hypoxia [141]. Similar effects have been shown in experimental models of Ewing's sarcoma [142], ovarican cancer [143] and gastric cancer [144]. In 2003 it was suggested by Hasselbalch that treatment with the tyrosine kinase inhibitor SU6668 could have beneficial effects in myelofibrosis [145], but no such study has as yet been published.

SU11248 is a recently described inhibitor with selectivity for PDGF receptors, VEGF receptors, FLT3 and KIT [146]. It has been shown to have significant effects in experimental tumor models of lung cancer [147] and breast cancer [148,149]. It has also been tried in a small phase I study on patients with FLT3-positive AML [150], where it was shown to successfully inhibit FLT-3 phosphorylation in AML blasts. The effects on pericytes or angiogenesis were not analyzed in this study, but the inhibition profile of this compound makes it an attractive candidate for treatment of myelofibrosis as well.

In the last part of the study (paper IV) we use mouse models of myelofibrosis to further clarify the molecular background to the disturbed angiogenesis in myelofibrosis. We can show that mice with an intrinsic (GATA-1^{low}) or extrinsic (TPO over-expression) alteration of the regulation of megakaryocyte maturation will result in increased MVD, abnormal vessel morphology and increased recruitment of pericytes. We can also show that the fibrogenic cytokine TGF-β1 is not mandatory for the development of pathological angiogenesis in TPO over-expressing mice, since mice lacking expression of TGF-β1 develop the same vascular abnormalities as their TGF-β1-expressing littermates. These results confirm the theory that dysregulated megakaryocyte maturation and an abnormal expression of angiogenic molecules play a vital part in the pathogenesis of this disease.

The specific molecular event that cause myelofibrosis remains to be elucidated, and as shown by the fact that two different genetic manipulations can cause a similar phenotype, it is very likely that there are more than one event. Research is now proceeding in this area, and as discussed in the introduction part, specifically over-and under -expressed genes have been defined. The finding that approximately half of myelofibrosis patients have a mutation in JAK2 might also give important clues. However, myelofibrosis is clinically a heterogeneous disease, and none of the described genetic changes is present in all patients with the clinical disease. We hope that as more insight is gained in the molecular regulation of megakaryocyte maturation, new candidate genes for the disease specific event in myelofibrosis might appear. Until then, therapy has to be directed towards the secondary changes of the disease.

The results of this study suggest that targeting cytokines and angiogenic molecules released from megakaryocytes might be a promising way. Targeting the pathological angiogenesis has the advantage of not causing great toxicity, since endothelial cells normally are quiescent, and are not affected by agents inhibiting endothelial cell proliferation.

The results of this study also suggest that pericyte recruitment is an important part of the disease and should be targeted as well. One advantage of inhibiting pericyte recruitment is that it could potentially destabilize vessels and make them more vulnerable to anti-angiogenic treatment. Further studies, more precisely defining the mechanisms of angiogenesis and pericyte-recruitment in myelofibrosis are needed to specifically design the cocktail of inhibitors that would possibly stop the development of the disease.

Conclusions

In this study we show that

- Angigogenic molecules over-expressed in many solid tumors are not over-expressed on pathological vessels in patents with PV or CML. Thus, these vessels have their own specific phenotype that remains to be defined.

- Tie-2, Cox-2 and glycodelin are expressed in bone marrow megakaryocytes by patients with PV and CML as well as of healthy controls. As megakaryocytes are accumulated in the bone marrow in the patients, the finding might be of importance for the development of the pathological angiogenesis seen in these disorders.

- Angiogenesis in myelofibrosis is characterized by morphologically aberrant vessels and increased pericyte recruitment.

- In two mouse models of myelofibrosis, with intrinsic or extrinsic changes in the regulation of megakaryocyte differentiation the same vascular abnormaities are seen. Therapy could thus be directed both towards the pathological endothelial cell proliferation and pericyte recrutment.

Future directions

To specifically target the pathological angiogenesis in patients with CMPD, the phenotype of these vessels have to be defined. This could be done by extending our strategy, using multiple antibodies and immunohistochemistry. However, this method is time consuming and has its limitations as described above. A more efficient approach would be to use DNA microarray analyses of cultivated bone marrow endothelium. In microarray analyses the expression of a defined set of genes (up to a hundred thousand on each array) is compared between patient and control tissue. This technique has been successfully used to determine specific gene expression of endothelial cells from different tissues [151] and could possibly identify genes differentially expressed on CMPD vessels. SAGE is another technique that could be used on cultivated endothelial cells. In this technique individual mRNA transcripts are associated with a tag and the abundance of each tag provides a quantitative measure of the transcript. SAGE is not dependent on preexisting databases (as in microarray studies) and therefore provides an unbiased view of gene expression profiles. SAGE has been used to identify tumor specific markers in colon cancer [33] and could possibly be used on bone marrow endothelium as well. Both microarray and SAGE are very sensitive techniques and demand a very high level of cell purity. There are also the inherent difficulties in using cultivated cells, as the expression of many genes is influenced by culture conditions and number of passages.

These disadvantages are avoided by using in vivo phage display. A library of peptides displayed on phage is created and injected into mice. These will then home to their specific target, which can be isolated and characterized. This technique was used to define aminopeptidase P as a specific marker for tumor endothelium in breast cancer [152] . The mouse models described in our study would be well fitted to use for the same purpose. The preferential strategy would probably be to use a combination of these techniques: to identify candidate markers on endothelial cells and vessels of mouse models, then to study the expression of these markers on human bone marrow biopsies by means of immunohistochemistry or northern blotting.

Having identified megakaryocytes and pericytes as important cells in the pathogenesis of myelofibrosis, new insights might be made by further studies of the mice models of myelofibrosis described here. The effects of thrombopoietin have already been evaluated in GATA-1^{low} mice [153]. In this study it was shown that exogenously administered TPO raised the GATA-1 content in megakaryocytes in GATA-1^{low} mice and it could halt some of the manifestations of the disease. If it can restore the pathological angiogenesis is still not investigated. In line with these results, these mice could also be treated with substances effecting the abnormal megakaryocyte cytokine expression (i.e. inhibitors of bFGF and VEGF signaling) and their effect on angiogenesis could be evaluated. Likewise, substances affecting pericyte proliferation and recruitment (inhibitors of PDGF or Tie-2 signaling) could also be studied. The very recent identification of a specific mutation in JAK2, present in peripheral blood granulocytes in the majority of PV patients and approximately half of ET and MF patients, suggests that signaling through this pathway is also important for the development of these diseases [103]. Lethally irradiated mice, transplanted with murine marrow cells where the JAK2 point mutation (Val617Phe) is introduced, develop persistent erythrocytosis[105]. Its effect on angiogenesis is still not investigated and such studies might bring even more insights into the molecular events underlying the pathological angiogenesis in these disorders.

Acknowledgements

Many people have directly or indirectly contributed to this thesis-I specifically want to thank

Jan Palmblad, my tutor. For being witty and stubborn, and never giving up.

Bihui Zhong, who faithfully helped me in the lab during the first tiresome years of my thesis work.

Anette Landström, who took over, always with a big friendly smile on her face

Kerstin Rosendahl and **Inger Wedin**, my extra mamas in the lab

Anette Hoffman for spending long hours at the confocal microscope with me

Rick Rogers, collaborator from Boston and car maniac

Björn Rozell, mouse specialist and provider of ideas and material

Professor Christer Betsholtz and **Anna Abrahamsson**, leading pericyte experts who provided invaluable help with pericyte stainings

Eva Lemaire for help with illustrations in this book

All collegues at the department of hematology and especially**; Katarina Leblanc, Lars Möllgård, Kajsa Larsson, Mats Merup, Christina Löfgren, Hans Hägglund** and **P-A Broliden** for being inspiring and taking good care of me when I was new at the department. The innebandyboys: **Stefan, Andreas, Martin, Björn** and especially **Hareth** who carried me off the court when my foot was broken

Per Oscarsson and my new collegues at the department of emergency medicine, for positive attitudes and for taking my night-calls in times of extreme time preassure

My dream team, constantly interrupting my serious research career with parties, snowboard trips and endless discussions**: Lisa, Johanna, Marianne, Minna** and **Nelli**

My family: **Gunilla, Karin** and **Askar, Erik** and **Malin** for always supporting me, especially when life plays its funny little tricks with me

This work has been supported by grants from The Swedish Medical Research Council (19X-05991, 71XS-13135), The Stockholm Cancer Society, King Gustav V:s Jubilee Fund, King Gustav V:s 80 Year Fund, The Swedish Association against Rheumatism, Karolinska Institutet, Swedish Foundation For Strategic Research and Huddinge University Hospital.

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PAPERS I-IV