Pericyte coverage of abnormal blood vessels in myelofibrotic bone marrows

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ABSTRACT

Background and Objectives

Myelofibrotic bone marrow displays abnormal angiogenesis but the pathogenic mechanisms of this are poorly understood. Since pericyte abnormalities are described on solid tumor vessels we studied whether vessel morphology and pericyte coverage in bone marrow samples from patients with myelofibrosis differed from that in samples from controls.

Design and Methods

We assessed the microvascular density (MVD), vessel morphology and pericyte coverage in bone marrows from 19 myelofibrosis patients and nine controls. We also studied the same parameters in two mouse models of myelofibrosis, with genetic alterations affecting megakaryocyte differentiation (i.e. one model with low GATA-1 expression and the other with over-expression of thrombopoietin).

Results

In myelofibrotic marrows, MVD was 3.8-fold greater than in controls ($p<0.001$) and vessels displayed 5.9-fold larger mean perimeters ($p<0.001$). MVD was 1.8-fold greater in *JAK2* V617F-positive than in negative patients ($p=0.026$). Moreover, 92±11 % of vessels in patients with myelofibrosis were pericyte-coated but only 51±20 % of vessels in controls ($p<0.001$). In the two mouse models of myelofibrosis caused by targeting megakaryocytopoesis, wide, pericyte-coated and morphologically aberrant vessels were detected. MVD was significantly greater in bone marrow and spleen samples from animals with myelofibrosis than in wild-type mice.

Interpretation and Conclusions

We conclude that angiogenesis is similarly abnormal in human and murine myelofibrosis with intense pericyte coating, presumably related to abnormal megakaryocytopoiesis.

Key words: angiogenesis, microvascular density, SMA, myelofibrosis, pericytes.

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Neoangiogenesis, manifested as, for example, high microvascular density (MVD), is a characteristic feature of solid tumors[1] and also of the bone marrow of hematologic malignancies.[2] Very high MVD has been demonstrated in chronic myeloid leukemia and myelofibrosis (MF).[3,4] Vessels in myelofibrotic bone marrow, as well as in solid tumors, are very tortuous and branched with abrupt changes in caliber.[3,5]

In solid tumors, the switch from quiescent to actively proliferating endothelial cells is thought to be driven by increased local concentrations of specific growth factors, e.g. vascular endothelial growth factor (VEGF). We reported previously that significantly higher numbers of VEGF-positive cells are present in MF bone marrow than in healthy marrow.[3] Angiopoietins 1 and 2 contribute, alone or in conjunction with VEGF, to promote proliferation, stabilization and remodeling of vessels.[6]

Recruitment of pericytes (PC) has recently emerged as an important concept in small vessel stabilization. The presence of these cells as a capillary-surrounding network, is believed to be a sign of vasculature maturity. PC are recruited to newly formed endothelial tubes by growth and chemotactic factors, e.g. platelet-derived growth factor-B (PDGF)-B. They are identified by means of their of expression of, for example, α-smooth muscle actin (SMA-α), the receptor for PDGF-B (PDGFR-β) and desmin.[7] PC appear to be involved in the control of hemodynamics and endothelial proliferation and differentiation.[7] PC have been demonstrated in developing arteries in embryonic chick bone marrow,[8] but very little is known about the occurrence of PC around human bone marrow vessels. If recruitment of PC to vessels fails, as in PDGF-B and PDGFR-β knock-out mice, vessels become tortuous with changes in caliber,[9] bearing a striking resemblance to vessels seen in MF. Moreover, a relative deficiency of PC has been seen in the tortuous vessels of glioblastomas and renal cell carcinomas while PC coverage in breast and colon cancer approaches that of normal vessels.[10] Pericyte morphology may be aberrant in tumor vessels.[11]

In this paper, we describe, for the first time, the presence of PC in human bone marrows and also in two mouse models of human MF. In the first model, mice over-expressing thrombopoietin after genetic manipulation develop, within 2-3 months, a disorder characterized by the same changes in bone marrow and peripheral blood as those occurring human MF and may develop acute myeloid leukemia.[12] These mice have increased plasma levels of transforming growth factor (TGF)-β1 and PDGF.[13] Although no mutations in the *TPO* gene nor marked alterations in thrombopoietin expression have been reported in patients with MF, an activating mutation of *JAK2* has been detected in 50% of patients with MF. Furthermore, more recently an activating *Mpl* mutation (W515L or W515K) has been found in 5% of the patients, providing some evidence that this disease is related to increased signaling of the TPO/Mpl pathway.[14] The second model concerns the transcription factor GATA-1, known to be important for erythroid and megakaryocyte differentiation. Mice with decreased expression of GATA-1 (GATA-1^{low}) will also develop a MF-like disorder, but more slowly than the mice over-expressing thrombopoietin,[15] with increased levels of TGF-β1, PDGF and VEGF in the bone marrow. Interestingly, megakaryocytes from patients with MF contain significantly reduced levels of GATA-1 protein.[16]

Here, we quantified PC in human and mice bone marrows to see whether PC deficiency may be associated with the vascular aberrations seen in the bone marrow of patients with MF.

Design and Methods

Patients

Bone marrow biopsies from 19 patients with MF (ten men and nine women; age range, 50 to 84 years; mean age, 66 years) and nine control subjects (eight men and one woman, 48 to 85 years; mean age 66 years),were retrieved as part of a routine diagnostic procedure. The patients were diagnosed and followed up at the Departments of Haematology, Karolinska University Hospital Huddinge, Stockholm, or Odense University Hospital, Denmark. For further clinical details, *see supplementary information*. The study was approved by the Ethical Committee of Huddinge University Hospital and the Karolinska Institutet, and carried out according to the guidelines of this Committee.

Mice

For details on the engineering of these mouse models *see supplementary information*.

Antibodies

For details on primary antibodies, directed against human and mouse antigens, *see supplementary information*.

Immunofluorescence and immunohistochemistry

Human specimens. To visualize vessels and PC by immunofluorescence, cells were labeled with CD34 or SMA-α antibodies by standard techniques. For details, *see supplementary information*. We also tried to visualize PC with antibodies directed against PDGFR-β and desmin using the same procedure as described there.

For details concerning VEGF , SMA-α and desmin immunohistochemistry, *see supplementary information*.

Mouse specimens. Staining for CD34 and CD31 on paraffin-embedded bone marrow or spleen samples was performed similarly, but to avoid non-specific staining due to mouse antibodies interacting with mouse tissue a biotinylated secondary mouse adsorbed rabbit anti-mouse antibody was used (Vector, Burlingame, CA, USA). To increase the signal, we used an enhancement step with biotinyl tyramid (Perkin Elmer TSA Biotin System kit). For further details, *see supplementary information*.

Double staining of mouse sections for CD34 and SMA was carried out with chromophore fast red, which is visi-

ble in both in ordinary and fluorescent light. For details, *see supplementary information*.

Analysis of vessel and PC morphology was done by fluorescent- and double laser confocal microscopy (Leica Q5501, Wetzlar, Germany).

Morphometric analyses

MVD and vessel perimeters in human bone marrow samples were determined by confocal microscopy. For details, *see supplementary information*.

Three-dimensional imaging

Tissue slabs, 30-50 μm thick, were attached to Superfrost Plus glass slides (VWR, West Chester, PA, USA), deparaffinized with Neoclear Xylene substitute (VWR), and rehydrated as described. For details on the staining procedure and the 3-D analysis, *see supplementary information*.

Statistical analyses

Analyses were performed with the Statistica™ software package using the Student´s t-test, χ^2-test and Spearman's correlation. Results are given as mean and SD values. *p* values <0.05 were considered statistically significant.

Results

Increased MVD and VEGF expression in bone marrow of patients with MF

We have previously shown that CD34 staining provides a reliable estimate of MVD, whereas estimates based on staining for von Willebrand factor or CD31 are sometimes confounded by mistaken identification of megakaryocytes and platelets as vessels.[20]

We and others have previously shown that virtually all blood vessels in human bone marrow are capillaries.[3,21] Consistent with previous results,[3] the mean MVD was significantly higher in the bone marrow from individuals with MF than in that from control subjects (Figure 1A). The number of cells expressing VEGF-A was also significantly higher in MF patients than in controls (Figure 1B). VEGF-expressing cells in the bone marrow of patients with MF have been characterized previously.[22] When we compared the MVD of *JAK2* V617F positive patients with that of patients without this mutation, the former had a significantly greater MVD (7.4±2 vs. 4.1±2, p=0.026) in bone marrow, but there was no significant difference for VEGF expression. We also compared MVD and VEGF expression in idiopathic MF (IMF), post-polycythemic myelofibrosis (PPMF) or post-thrombocythemic MF (PTMF) patients (*see supplementary material*), but no significant differences were found (*data not shown*). Moreover, there was no statistically significant correlation between MVD and VEGF-expressing cells in all patients with MF.

Bone marrow samples from patients with MF secondary to the non-myeloproliferative disorders Sjögren's syndrome and neutropenia due to large granular lymphocyte (LGL) syndrome (*see supplementary material*) also displayed increased MVD (10 and 16.8 vessels/hpf, respectively) and a high level of VEGF expression (37 and 62 cells/hpf).

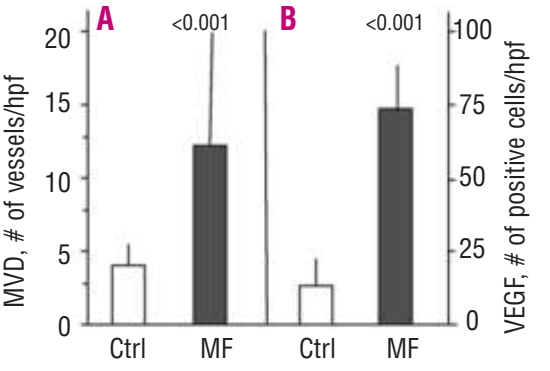

Figure 1. Increased MVD and VEGF expression in bone marrow of MF patients. A. MVD was determined by counting all vessels in five high power fields (hpf) per section of bone marrow from MF patients and control subjects (Ctrl). B. The number of cells expressing VEGF-A per hpf was also determined for five hpf per section of bone marrow from MF patients and controls.

Aberrant morphology of vessels in bone marrow from patients with MF

The mean perimeter of vessels in the bone marrow of individuals with MF was significantly larger than that of vessels of control subjects (113±59 vs. 19±9 μm, p<0.001). Consistent with previous results,[3] vessels in the bone marrow of MF patients had an aberrant morphology with a highly variable caliber. Whereas all vessels in control marrows had a perimeter of <100 μm, a substantial proportion (17.2%) of vessels in the bone marrow of MF patients had a perimeter of ≥100 μm (Figure 2). There was no significant difference in mean perimeters of vessels from *JAK2* V617F positive or negative patients. In addition, no differences were found between vessels from patients with IMF, PPMF or PTMF.

In contrast to patients with MF due to a myeloproliferative disorder, patients with MF secondary to Sjögren syndrome or LGL-associated severe neutropenia did not show any morphological abnormalities of bone marrow vessels.

Identification of PC in human bone marrow by confocal microscopy

Antibodies to SMA-α specifically labeled PC in thick sections of bone marrow from control subjects and MF patients (Figure 3). Vessels exhibited similar patterns of staining with these antibodies, with the exception that the number of vessel profiles associated with PC-like cells appeared greater in MF patients than in controls (*see below*). PC in all specimens typically exhibited a rounded mononuclear profile in cross-sections of the vessel lumen (Figure 3). PC processes were apparent as finger-like cytoplasmic projections wrapped around the vessel perimeter in three-dimensioned reconstructions of vessels oriented longitudi-

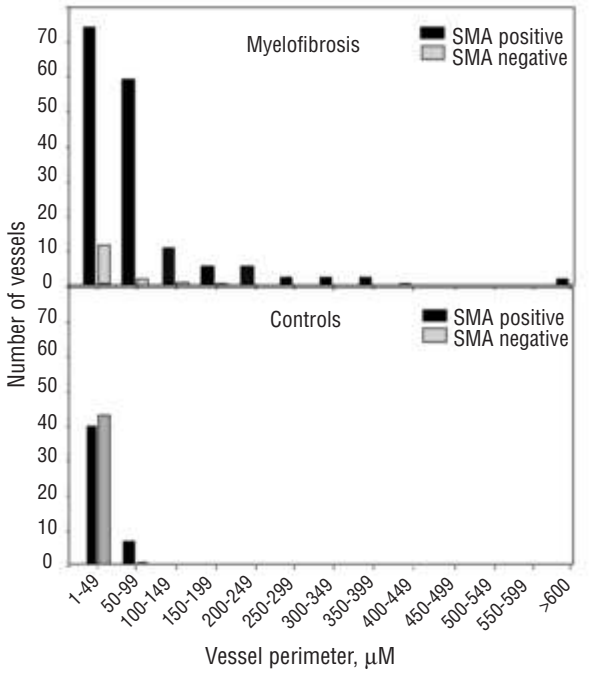

Figure 2. Shift in the size distribution of blood vessels in bone marrow of patients with myelofibrosis. The perimeters of 185 vessels in the bone marrow of MF patients and of 90 vessels in the marrow of control subjects were determined. Vessels were classified as PC-coated (positive for both SMA-α and CD34) or non-coated (negative for SMA-α, positive for CD34). In control marrow, the perimeter of all vessels was <100 µm, with that of most being <50 µm. In MF marrow, although most vessels were in the same size range as those in control marrow, vessels of much larger size, with perimeters up to 900 µm, were present.

nally. PC of MF patients and controls appeared morphologically similar.

We also used an antibody to human desmin to identify PC. However, the antibody stained only a few bone marrow cells with no relation to vascular structures in either MF patients or control subjects (*data nor shown*). A positive control for this analysis revealed that similarly prepared sections of human skeletal muscle showed positive staining for desmin in cells associated with muscle fibers as well as in perivascular cells (*data not shown*), indicating that the staining procedure was appropriate for the intended purpose.

Antibodies to PDGFR-β stained perivascular cells only in the bone marrow of MF patients, not in that of control subjects. The staining was of relatively low intensity and only a minor proportion (3%) of recognizable vessel structures was positive. The PDGFR-β-expressing cells surrounded capillaries and double staining revealed that these cells were also weakly positive for SMA-α (*Figure 8, in the Supplement*). Given that SMA-α staining has been found to reliably identify PC in most tissues, whereas reactivity of these cells with antibodies to desmin or to PDGFR-β is more variable, being both tissue and vessel specific,[23] we used SMA-α as the PC marker for subsequent analyses.

Increased proportion of PC-coated vessels in myelofibrotic bone marrow

Bone marrow sections double stained for CD34 and SMA-α were then analyzed for PC coverage of vessels. For this analysis, PC were defined as SMA-α-positive cells with cytoplasmic extensions that surrounded CD34-positive endothelial cells of vessels with a clearly visualized lumen. We analyzed approxinately ten randomly selected cross-cut vessels per slide for each MF patient or control subject and determined the numbers of PC-coated and non-coated vessels. Whereas only 51±20% of vessels in the bone marrow of control subjects were coated with PC, 92±11% of vessels in the marrow of MF patients were so ($p<0.001$). The proportion of PC-coated vessels did not differ significantly between *JAK2* V617F positive and negative patients. Likewise, there was no difference in PC coverage between patients with primary IMF, PPMF and PTMF.

In the two patients with MF due to other diseases, there was no increase in pericyte coverage, rather the opposite. Less than 1% of the vessels were PC-coated in both patients.

Increased perimeter of PC-coated vessels in myelofibrotic marrow

We then examined whether PC coverage was specific to vessels of a certain size. In bone marrow from control subjects, the perimeter of PC-coated vessels was significantly larger than that of non-coated vessels (25±20 vs. 14±10 µm, $p<0.001$). PC-coated vessels in myelofibrotic bone marrows had a mean perimeter of 87±62 µm and were, thus, significantly larger ($p<0.001$) than those of control marrow (see also Figure 2). Non-coated vessels were also signifi-

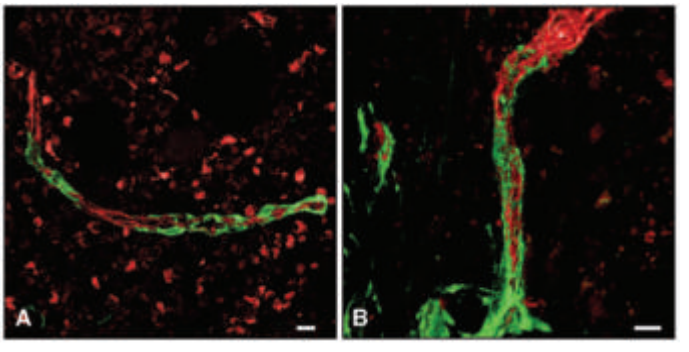

Figure 3. Altered PC morphology in bone marrow. Confocal three-dimensional reconstructions of vessels were generated from serial optical sections of bone marrow biopsies. A vessel from a control subject is shown in (A) and one from a MF patient in (B). PC are positive for SMA-α (green) and typically exhibit a rounded mononuclear profile with finger-like cytoplasmic projections that encapsulate the perimeter of vessels positive for CD34 (red). Scale bar, 10 µm.

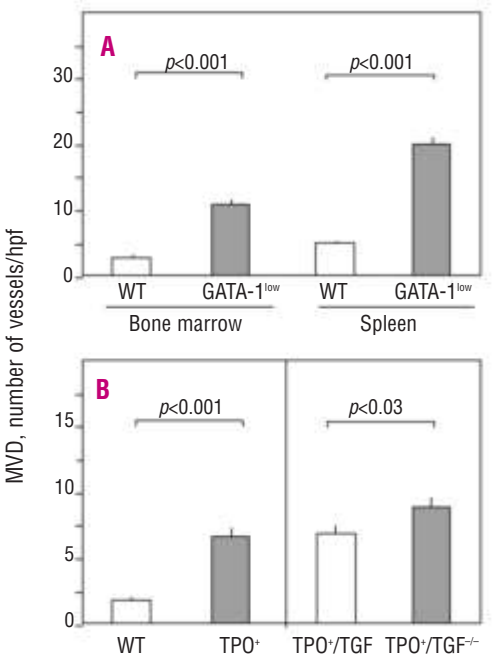

Figure 4. MVD in different mouse models of MF. Vessels in bone marrow and spleen of wild-type (WT) and GATA-1^{low} mice were stained with an antibody directed against CD34, and MVD was calculated for each section (*panel A*). GATA-1^{low} mice showed significantly greater MVD in both the bone marrow and spleen. MVD was also significantly greater in the bone marrow from mice over-expressing thrombopoietin (TPO) (*panel B*). In a second experiment, lethally irradiated mice were transplanted with hematopoietic cells from TGF-β null mice over-expressing TPO or from TPO-over-expressing mice only. As seen in panel B, the absence of TGF-β did not restore the TPO-induced angiogenesis. On the contrary, these mice showed slightly greater MVD in the bone marrow.

cantly wider in MF patients than in controls (mean perimeter 49±32 vs. 13±6 μm, respectively, $p<0.001$). There were no significant differences in mean perimeter, in either in PC-coated or uncoated vessels, between *JAK2* V617F positive and negative patients.

Some vessels in MF patients appeared to be devoid of endothelial cells within the tube formed by PC (*Figure 9, in the Supplement*).

GATA-1^{low} and thrombopoietin over-expressing mice have increased MVD in bone marrow and spleen

Vessels were visualized by staining endothelial cells for CD31 and CD34. Both markers stained vessels in GATA-1^{low} mice, but the CD31 antibody did not stain vessels in mice over-expressing thrombopoietin. Again, CD31 has the disadvantage of staining megakaryocytes, which could jeopardize the specificity of the MVD analysis, in this very megakaryocyte-rich environment. We, thus, chose CD34 as the marker of vessels for MVD determinations.

The MVD in bone marrow samples from GATA-1^{low} mice was 3.6-fold greater than that of the wild-type mice. The difference in the spleen was even more striking (Figure 4A). As also seen in human MF, the GATA-1^{low} bone marrow vessels were dilated, tortuous, with changing calibers (Figure 5).

The mean bone marrow MVD in thrombopoietin over-expressing mice was also significantly greater than that in the wild-type animals (Figure 4B) and the bone marrow vessels showed the same morphological abnormalities as those in the GATA-1^{low} mice (Figure 6B).

The abnormal angiogenesis in thrombopoietin over-expressing mice is not related to TGF-β1 or the development of MF

To investigate the significance of TGF-β1 in the development of pathological angiogenesis in thrombopoietin over-expressing mice, lethally irradiated wild-type hosts were engrafted with bone marrow stem cells from homozygous TGF-β1 null (TGF-β1$^{-/-}$) or WT littermates, infected with a retrovirus encoding the murine thrombopoietin protein. This procedure had to be used since TGF-β1$^{-/-}$ mice die soon after birth. In the absence of TGF-β1 no bone marrow or spleen fibrosis developed, but all other hallmarks of a myeloproliferative disorder remained,[12] i.e. thrombocytosis, leukocytosis, splenomegaly and increased numbers of blood myeloid progenitors.

The MVD in bone marrow of mice not expressing TGF-β1 was slightly increased compared to that of mice with normal TGF-β1 (Figure 4B). The vessels showed the same, or even worse, morphological aberrations as those seen in mice over-expressing thrombopoietin with normal TGF-β1 levels (Figure 6C).

The abnormal vessels of GATA-1^{low} and thrombopoietin over-expressing mice are coated with PC

To visualize PC we used antibodies directed against SMA-α and desmin. Desmin did not stain any cells in the mouse bone marrow or spleen, but SMA-α identified a population of cells surrounding vessels in wild-type mice. We, thus, used SMA-α to study PC in the genetically modified mice. We analyzed approximately 30 randomly

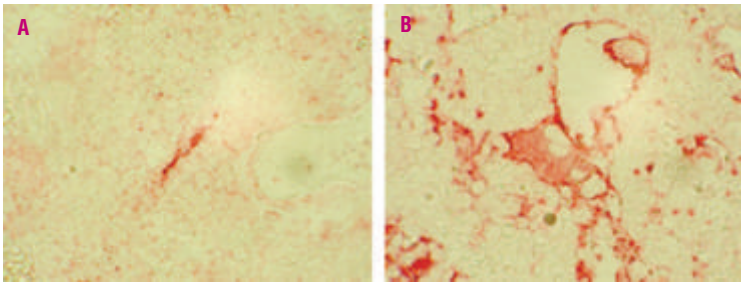

Figure 5. Vessel morphology in the bone marrow of wild-type and GATA-1^{low} mice. Representative bone marrow vessel from a wild-type (panel A) and GATA-1^{low} mouse (panel B) immunostained for CD34. The vessels from the GATA-1^{low} mouse were generally wide and tortuous, resembling vessels in human MF. bar: 10 μm.

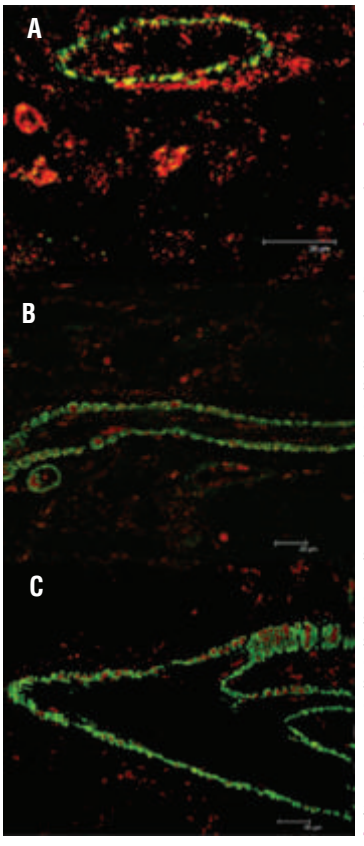

Figure 6. PC expression in the bone marrow of wild-type mice compared to that in thrombopoietin over-expressing mice with or without expression of TGF-β1. While most PC-coated vessels in wild-type mice were relatively small and regularly shaped (panel A), those of thrombopoietin over-expressing mice were gathered in clusters and were grossly dilated (panel B). Thrombopoietin over-expressing mice, lacking expression of TGF-β1 in the bone marrow, also developed these hot spots of PC-coated vessels. As seen in panel (C) some of these vessels had aberrant pericyte morphology, with PC completely surrounding the endothelial cells in a smooth-muscle-cell fashion. Scale bar=20 μm.

selected cross-cut vessels per slide and determined the numbers of pericyte-coated and non-coated vessels.

The relative numbers of PC-coated vessels were lower in the bone marrow of GATA-1^{low} mice than in controls (71% of all bone marrow vessels were PC-coated in wild-type bone marrow vs. 47% in GATA-1^{low} mice), but the difference was not statistically significantly different (p>0.05). In the spleen, the relative numbers were comparable (37% in wild-type vs. 41% in GATA-1^{low} mice). We observed that it was primarily the enlarged abnormal vessels that were PC-coated (Figure 7).

In thrombopoietin over-expressing mice, the large dilated vessels were covered with PC, and the PC themselves showed variable morphology. The normal appearance, with finger-like projections embracing the endothelial cells, sometimes acquired more smooth muscle cell-like features (Figure 6). However, the relative number of PC-coated vessels was almost the same in wild-type and thrombopoietin over-expressing mice (32% vs. 38%, respectively).

Absence of TGF-β1 does not influence PC coverage

The same morphological characteristics, i.e., abnormally dilated and irregularly branching vessels, as seen in human MF, GATA-1^{low} or thrombopoietin over-expressing mice, were also apparent in thrombopoietin over-expressing mice reconstituted with TGF-β1$^{-/-}$ hematopoietic cells; the percentage of the total number of vessels that was PC-coated in mice, with or without TGF-β1, was also the same (18%).

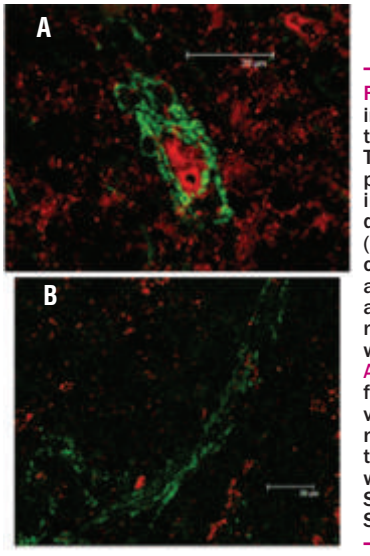

Figure 7. PC expression in the spleen of wild-type and GATA-1^{low} mice. To visualize vessel morphology, double staining, with antibodies directed against CD34 (recognizing endothelial cells, red color) and antibodies directed against SMA-α (recognizing PC) was used. In wild-type mice (panel A), PC could be identified surrounding normal vessels. In GATA-1^{low} mice (panel B) most of the abnormal vessels were surrounded by SMA-α staining cells. Scale bar=20 μm.

Discussion

We show here that PC cover blood vessels in bone marrows of both healthy humans and individuals with MF, a disease characterized by disturbed angiogenesis. Unexpectedly, the extent of PC coverage of marrow vessels in MF patients (IMF, PPMF and PTMF alike) was markedly greater than that in normal subjects.

The V617F mutation in the tyrosine kinase JAK2 has been shown to be present in approximately 50% of patients with MF. Hypothetically, a constitutively active kinase might fuel not only the myeloproliferative process but also a not yet identified pro-angiogenetic pathway. Experimentally, erythropoietin and granulocyte-macrophage colony-stimulating factor signaling through JAK2 have been shown to induce an angiogenic response in cultured endothelial cells.[24,25] Our results may support this concept, since patients with the JAK2 mutation had significantly greater MVD than those without. Further studies on larger series of patients are needed to determine the role of the JAK2 mutation in angiogenesis.

The aberrant vascular architecture in bone marrow of MF patients resembles that in naturally occurring or experimentally-induced tumors, in which vessels form in response to an increased local expression of, for instance, VEGF.[26] A similarly disorganized vessel architecture characterizes patients with a mutation in the gene for the angiopoietin receptor Tie-2.[27] Consequently, we hypothesized that abnormal angiogenesis in MF might be mediated by aberrant signaling through VEGF or angiopoietin pathways. This hypothesis was consistent with the previous findings of Lundberg *et al.*[3] (and partly Wrobel[28] and Ni *et al.*[29] however, not by Ho *et al.*[22]) that MF patients displayed increased cellular bone marrow expression of VEGF relative to controls. Our study confirmed this finding in a new cohort of MF patients, but did not detect any correlation between MVD and VEGF expression. The relation of

VEGF to MVD and vascular architecture in MF is, therefore, complex and needs further evaluation. Nonetheless, we[20] and others[21] previously found that Tie-2 expression in bone marrow vessels of patients with polycythemia vera, chronic myeloid leukemia, or MF did not differ from that in bone marrow vessels of healthy controls. As described for certain tumors with aberrant vessels,[10] deficient PC coverage of vessels is a new candidate to explain the vessel abnormalities in MF.

Both endothelial cells and PC are required for the formation of functional blood vessels. The importance of PC has only recently emerged.[31] PC partially cover the abluminal endothelial surface and are thought to participate in activities related to angiogenesis, including sensing the physiological needs of the tissue and hemodynamic forces within the vessel, deposition and degradation of extracellular matrix, and regulation, in a paracrine manner, of endothelial cell proliferation and differentiation. The extent of PC coverage varies widely among capillary beds of different tissues.[32] In general, vessels engaged in transport of gases and nutrients have few PC, whereas vessels in neural tissues have the highest PC density.[32] We now show that human and mouse bone marrow vessels contain PC.

The identification of PC is problematic because no single marker distinguishes them. Given that no data were available on the PC coverage of vessels in normal human bone marrow, we examined the expression of three widely used markers of these cells in humans: desmin, PDGFR-β, and SMA-α. Of these proteins, cells that were located in close proximity to bone marrow vessels and that morphologically resembled PC, expressed only SMA-α and PDGFR-β. Given that only a small proportion of the perivascular cells were PDGFR-β positive and only found in myelofibrotic marrow, we identified PC as SMA-α-positive cells that were present on the abluminal endothelial surface of capillaries. Our data suggest that PC of normal bone marrow differ phenotypically from those in some other tissues in that they do not consistently express desmin or PDGFR-β.

Unexpectedly, the extent of vessel coverage by PC was markedly greater in bone marrow from MF patients than in that from normal individuals. Whereas vessels with a perimeter of ≥100 μm were not detected in normal marrow, such vessels were present in the marrow of MF patients and were coated with PC. These findings suggest that the aberrant vessel morphology apparent in the marrow of MF patients is not due to a lack of PC but rather is associated with increased PC coverage. Further studies of the role of PC in MF are needed in order to understand interactions with endothelial cells.

The mechanisms for increased PC coverage of abnormal vessels in MF are not known. Since PC are believed to be recruited by PDGF-B, normally secreted from endothelial cells and megakaryocytes,[23,33] this molecule is an obvious candidate. This assumption is supported by the findings that many of the reactive changes apparent in MF may be caused by dysmegakaryopoiesis and the release of megakaryocyte growth factors, such as PDGF-B, in bone marrow.[34,35] Other chemotactic molecules must also be considered.

The pivotal role of altered megakaryocyte differentiation in the pathogenesis of MF has been demonstrated by the fact that genetically altered mice, with either increased expression of thrombopoietin or low expression of GATA-1, develop a myeloproliferative syndrome with many of the same morphological changes as those present in the human disease. Consequently, we also studied angiogenesis and PC coverage in three mice models.

Here, we show that mice with low expression of GATA-1 or overexpression of thrombopoietin develop increased angiogenesis both in bone marrow, and even more strikingly so, in the spleen. The vessels show the same morphological abnormalities as those in human MF with changing calibers and abnormal branching. Also, the major PC-recruiting cytokine, PDGF-B, is over-expressed in the extracellular fluid of GATA-1^{low} and thrombopoietin over-expressing mice.[15] We, therefore, wondered whether the angiogenic changes in these mice were secondary to dysregulated PC recruitment. Interestingly, it was primarily the large, abnormal vessels of the genetically modified mice that were PC-coated, mimicking the situation in human MF.

We have, therefore, shown the presence of abnormal angiogenesis in GATA-1^{low} as well as in thrombopoietin over-expressing mice, suggesting that megakaryocyte dysregulation is an important step in this process. We were also able to study angiogenesis in the bone marrow of thrombopoietin^{high} mice reconstituted with TGF-β1$^{-/-}$ hematopoietic cells. In this model, there is no TGF-β1 of hematopoietic origin but there is still some TGFβ1 synthesized by the stroma. These mice develop a myeloproliferative syndrome similar to that of mice with normal TFG-β1, but without bone marrow fibrosis.[12] They have significantly increased MVD and an abundance of pathological vessels in the bone marrow, suggesting that the TFG-β1 synthesized by hematopoietic cells is not pivotal for the development of pathologic angiogenesis. Thus, PDGF might be the crucial cytokine responsible for this abnormal angiogenesis. This would also suggest that fibrosis *per se* is not a sufficient event for formation of pathological vessels, a notion that is further supported by the finding of normal vessel morphology and PC coverage in patients with MF secondary to Sjögren's syndrome and LGL (although MVD was increased in both).

The treatment of MF remains unsatisfactory. Our findings suggest that the pathological vessel morphology seen in MF is not due to a lack of PC but rather associated with a high number of these cells. Moreover, we demonstrated a novel concept in the pathogenesis of MF, whereby the myeloproliferative disorder *per se* is for the abnormal angiogenesis and not directly the MF. Specific targeting of PC emerges as a potential new treatment option, particularly in view of the notion that current angiostatic drugs might have less effect on mature vessels.[36,37]

Authors' Contributions

EZ designed and performed the research, analyzed the data and wrote the paper; AMV, ARM, MW, MT and HH contributed vital new reagents or analytical tools; RD performed research; RR designed the research and contributed vital new reagents or analytical tools; JP designed the research, analyzed the data and wrote the paper.

Conflict of Interest

The authors reported no potential conflicts of interest.

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