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Review

Bone marrow angiogenesis: methods of quantification and changes evolving in chronic myeloproliferative disorders

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Summary. Until now little information is available about bone marrow (BM) angiogenesis in chronic myeloproliferative disorders (CMPDs). Amongst the various immunohistochemical markers for endothelial cells CD34 and CD105 have proven to be most reliable since they exhibit no relevant co-staining. Determination of vascularity has to include pathophysiological aspects of perfusion. Therefore, quantification of the microvascular density (MVD) by the so-called hot spot method has to be improved by parameters that characterize blood flow more properly like microvessel area (luminal distension), shape (form factor), tortuosity, and branching (maximal vessel length). In comparison to the normal BM chronic myeloid leukemia (CML) revealed a significant increase in MVD which was functionally associated with elevated levels of angiogenic cytokines. Structure of vessels was significantly altered by showing an enhanced irregularity of shape and tortuosity and increase in fibers was conspicuously accompanied by a higher degree of MVD. Contrasting the group of patients with Imatinib (STI571) therapy interferon failed to reduce the number of vessels. Following bone marrow transplantation a significant enhancement of the MVD was found in the early post-transplant period, but after about 6 months normalization occurred. Anomalies of microvascular architecture were easily demonstrable by three-dimensional reconstruction and consisted of a complex branching network of irregular shaped sinuses. Chronic idiopathic myelofibrosis displayed a significant increase in the MVD only in the advanced fibrosclerotic stages. This feature was accompanied by an enhanced luminal distension and tortuosity, thus contrasting the prefibrotic and early fibrotic phases of this disorder. Similar to CML a relationship between evolving myelofibrosis and change in vascular architecture was encountered. This feature may present a possible target for future anti-angiogenic therapy. In essential thrombocythemia there is only a mild increase in MVD detectable while in polycythemia vera besides an enlarged number, a luminal dilation due to the densely packed erythrocytes is recognizable.

In conclusion, contrasting the usually applied quantification technique more elaborate morphometrical methods are warranted to obtain a better insight into the vascular architecture of the BM. In CMPDs angiogenesis is significantly associated with the evolution of myelofibrosis and may be altered by therapeutic regimens probably due to changes in cytokine release.

Key words: Angiogenesis, Chronic myeloproliferative disorders, Microvessel density, Sinusoidal architecture, Myelofibrosis, 3D-reconstruction

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Introduction

A wealth of data has been accumulated about the pivotal role of angiogenesis for tumor growth, including its potency for invasion, metastasis and progression (Folkman, 1971; Hanahan and Folkman, 1996; Fox, 1997; Weidner, 1999; Carmeliet and Jain, 2000). In this regard microvessel density (MVD) has come into the focus of scientific interest, because this feature was speculated to reflect the angiogenic capacity of neoplastic cells and consequently was assumed to mirror aggressiveness of the disease process (Fox, 1997). Consequently angiogenesis has been established as an independent prognostic factor for carcinomas of the large bowel, breast, cervix, lungs, prostate gland, esophagus and urinary bladder (Weidner et al., 1991, 1993; Bossi et al., 1995; Fox et al., 1995; Vermeulen et al., 1996; Pavlopoulos et al., 1998; Carmeliet and Jain, 2000; Guidi et al., 2000; Korkolopoulou et al., 2001). Although within the bone marrow (BM) microenvironment the endothelial cell layer was known to act as gatekeeper controlling the trafficking and homing of hematopoietic progenitors by preserving a

steady state hematopoiesis (Nagaoka et al., 1986; Abboud, 1995; Rafii et al., 1995, 1997), only recently the importance of vascularization for neoplasias of the lymphohematopoietic systems was recognized (Bertolini et al., 2000; Mangi and Newland, 2000; Moehler et al., 2001; Rajkumar et al., 2002a,b; Stasi and Amadori, 2002; Aguayo et al., 2003). In this regard the majority of investigations are in keeping with the assumption that in acute and chronic leukemias including myelodysplastic syndromes microvessel density exerts a significant impact on the expansion of the neoplastic cell clone (Perez-Atayde et al., 1997; Aguayo et al., 2000; Di Raimondo et al., 2000b; Garland et al., 2000; Hussong et al., 2000; Kini et al., 2000; Padro et al., 2000; Albitar, 2001; De Bont et al., 2001; Kini et al., 2001; Korkolopoulou et al., 2003b). Similar findings were reported for multiple myeloma (Rajkumar et al., 1999; Sezer et al., 2001; Vacca et al., 2001; Pruneri et al., 2002), systemic mastocytosis (Wimazal et al., 2002) and chronic myeloproliferative disorders - CMPDs (Reilly et al., 1985; Thiele et al., 1992; Lundberg et al., 2000; Mesa et al., 2000; Di Raimondo et al., 2001). Prognostic implications of this striking phenomenon were especially postulated for multiple myeloma (Vacca et al., 1994; Ribatti et al., 1999; Rajkumar et al., 2002a), acute leukemia (Di Raimondo et al., 2000a) and myelodysplastic syndromes (Pruneri et al., 1999). It is noteworthy that angiogenesis is generated by release of certain mediators like the vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and the platelet derived growth factor (PDGF) secreted by the tumor itself or bystander cells like macrophages, mast cells, lymphocytes and fibroblasts (Millauer et al., 1993; Carmeliet and Jain, 2000; McMahon, 2000). In this context a stimulation of VEGF secretion has been significantly correlated with a worsening of prognosis in solid tumors (McMahon, 2000). It was postulated that this effect of VEGF is related to the inhibition of apoptosis of endothelial cells and their increased potency for trafficking and migration (Ferrara and Gerber, 2001; Ferrara, 2002; Giles, 2001; Glade-Bender et al., 2003). Regarding hematological malignancies VEGF was shown to be expressed in a variety of cultured or isolated human leukemic cell lineages (Fiedler et al., 1997; Bellamy et al., 1999; Verstovsek et al., 2002a,b) by paracrine and autocrine signal transduction pathways (Moehler et al., 2001). This complex pathomechanism may comprise a direct stimulation of endothelial cells (paracrine loop) resulting in proliferation, sprouting and release of growth factors and on the other hand, an autocrine stimulation by inhibition of apoptosis and promotion of growth (Fig. 1), finally leading to an expansion of the neoplastic cell clone (Mangi and Newland, 2000; Moehler et al., 2001). According to these findings, microvessel density in the BM may turn out to be a future target for innovative anti-angiogenic and anti-leukemic therapeutic strategies and therefore warrants a scrutinized and systematic investigation (Beranek, 2001; Dickson and Shami, 2001; Thomas et al., 2001; Singhal and Mehta, 2002).

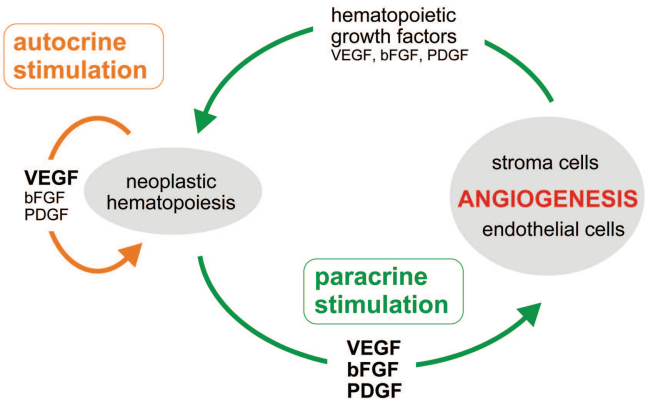


Fig. 1. Dynamics of angiogenesis in hematological neoplasias: angiogenic factors secreted by neoplastic cells promote cell growth and inhibit apoptosis (autocrine stimulation). On the other side, these angiogenic mediators can also stimulate endothelial cell proliferation and enhance the production and release of hematopoietic growth factors (paracrine stimulation).

Labeling of endothelial cells

In the past years a striking variety of techniques have been applied to determine the frequency of vascular structures (sinusoids, capillaries, large vessels) in the BM (Vermeulen et al., 1996; Kvasnicka and Thiele, 2002). First of all, a specific stain is required to visualize distinctively the endothelial cell layer for a proper assessment of microvascularity. The immunohistochemical methods that were usually involved in these investigations have to be discussed very critically, since some markers may detect different constituents of the BM and therefore also various aspects of vascular structures characterizing the stroma compartment (Table 1). Contrasting the situation in solid tumors it seems to be mandatory to avoid antibodies like the frequently used Factor VIII (Little et al., 1986; Aguayo et al., 2000) due to the relevant co-staining of other prominent BM cells, i.e. megakaryocytes that may be abundant in CMPDs (Mesa et al., 2000; Kvasnicka and Thiele, 2002). Other antibodies like anti-collagen IV lable only a certain amount of vessels, i.e. those exhibiting basement membrane material and consequently fail to react with very small sinusoids (Reilly et al., 1985; Thiele et al., 1992, 1994; Kvasnicka et al., 1994; Kvasnicka and Thiele, 1995). On the other hand, besides CD105 (Akagi et al., 2002), CD34 has become very popular concerning the identification of endothelial cells in the majority of studies dealing with BM angiogenesis (Pruneri et al., 1999; Mesa et al., 2000; Rajkumar et al., 2002a,b; Wimazal et al., 2002; Korkolopoulou et al., 2003b; Kvasnicka et al., 2004a,b). Staining reaction is very reliable with CD34, although few and dispersed progenitor cells are co-stained which are easily

distinguishable from endothelial cells by morphology (Kvasnicka and Thiele, 2002; Kvasnicka et al., 2004b).

Evaluation of angiogenesis

It seems to be reasonable that quantification of MVD and other parameters characterizing vascular architecture should be based on morphometric analysis (Kvasnicka and Thiele, 2002; Korkolopoulou et al., 2003a,b). This is a crucial issue, because when studying sections of solid tumors a number of investigators have discussed very controversially this impending problem (Weidner et al., 1991; Fox et al., 1995; Hansen et al., 1998). For reasons of practicability, so-called hot spots, i.e. areas exhibiting the highest MVD were firstly identified at low magnification (x100) in the tissue sample and subsequently final assessment was limited to a (semi-) quantification of these foci at high power (x400) view (Weidner et al., 1991). This technique was used with the aim to neutralize the possibility of a heterogeneous distribution of vascular structures within the neoplastic tissue (Vermeulen et al., 1996). Moreover, it has been argued that areas with intense neovasularization may reflect the close and functionally important interaction between neoplastic cells and ensuing proliferation of the endothelium associated with tumor growth and spread (Hanahan and Folkman, 1996; Carmeliet and Jain, 2000). Although a conflict of opinion continues concerning the reliability of counting hot spots (Martin et al., 1997; Hansen et al., 1998) this method was also applied to quantify BM vessels (Lundberg et al., 2000; Mesa et al., 2000; Moehler et al., 2001). However, when dealing with hematopoietic tissue significant differences in comparison to solid tumors should not be overlooked. It is mandatory to realize that in the BM no homogeneous neoplastic cell population is present and that the interstitial compartment has a significant influence on the development of vessels which include many small sinusoids (Moehler et al., 2001). Furthermore, depending on the age of patients and subtype of hematological malignancy a various amount of adipose tissue, fibrous matrix or edema may be a conspicuous finding. As a consequence, quantification of MVD has to be performed by regarding cellularity, i.e. hematopoietic area (Thiele et al., 1992; Kvasnicka et al., 2004b). Finally, it has to be considered that changes in angiogenesis are necessarily associated with alterations in circulation as a prerequisite for enhanced cell proliferation and trafficking eventually leading to tumor growth and metastatic spread. To get a better insight into functional changes of the blood flow, measurements focussed on the frequency of vascular structures in a certain section plane, but especially in the selected so-called hot spots (Weidner et al., 1991), are believed to be a very crude method. Concerning the pathophysiology of circulation other factors that are known to exert a modifying influence on perfusion have to be additionally regarded. These include the determination of luminal distension or relative area occupied by the blood vessels (Aguayo et al., 2000, 2003) and in particular tortuosity (deviation of a circular perimeter) and branching (maximal length) of a vessel (Thiele et al., 1992; Kvasnicka and Thiele, 2002; Korkolopoulou et al., 2003a,b) as illustrated in Figure 2. An even more advanced technique is a three-dimensional computer-assisted reconstruction of the vascular architecture by using serial sections. However, this method is limited to certain specimens and areas, because appropriate realization of these images is very time-consuming and burdensome (Kvasnicka et al., 1994; Thiele et al., 1994; Kvasnicka and Thiele, 1995; Lundberg et al., 2000).

Chronic myeloproliferative disorders (CMPDs)

Chronic myeloid leukemia

In Philadelphia chromosome-positive (Ph^{1+}) chronic myeloid leukemia (CML) the MVD is significantly increased by about a two-fold degree in comparison with the normal BM (Aguayo et al., 2000; Lundberg et al., 2000; Korkolopoulou et al., 2003b). According to three-dimensional imaging the newly-formed vessels were shown to exhibit striking irregularities and an abnormal branching with varieties in luminal distension that suggested a significantly altered blood flow (Lundberg et al., 2000). In the context of neo-angiogenesis in CML, plasma levels of VEGF and bFGF were also found to be increased (Aguayo et al., 2000; Lundberg et al., 2000).

Table 1. Antibodies frequently applied to identify vascular structures in the bone marrow in chronic myeloproliferative disorders.

ANTIBODY	SPECIFICITY	AUTHORS
Factor VIII	Prominent staining of megakaryocytes; not all endothelial cells are labeled	Lundberg et al., 2000
CD31	Positive reaction with megakaryocytes, thrombocytes and plasma cells; not all sinusoids are stained	Lundberg et al., 2000
UEA-1	Significant reaction with megakaryocytes, erythroid precursors and stroma cells	Lundberg et al., 200
Collagen-IV	Only vessels with basement membrane are stained, no reaction with small sinusoids	Reilly et al., 1985; Thiele et al., 1992; Kvasnicka et al., 1994
CD105	Specific and reliable marker of all endothelial cells	Kvasnicka and Thiele, 2002; Pruneri et al., 2002
CD34	Specific and reliable labeling of endothelial cells, co-staining of progenitor cells	Mesa et al., 2000; Kvasnicka and Thiele, 2002

Preliminary studies on small series of CML patients suggest that higher levels of VEGF are associated with an unfavorable prognosis (Verstovsek et al., 2002a,b). On the other hand, possible relationships between microvessels and other stroma constituents like reticulin and collagen fibers (myelofibrosis) have not been investigated so far and there is also no study on changes following clearly defined therapeutic regimens (i.e. drug treatment and transplantation).

To compare and especially to extend the data of these former studies we evaluated the BM vascularity in a first series of 85 patients with chronic phase Ph^{1+} CML. Only patients with more than one sequential BM biopsy at standardized follow-up check points (median interval 13 months) and without any prior treatment were regarded (Kvasnicka et al., 2004b). Patients received either monotherapy with Interferon-α (IFN), Hydroxyurea (HU), or a combination of IFN and HU (Hehlmann et al., 2000; Kantarjian et al., 2003; Silver et al., 2003). Moreover, we recruited patients who obtained a specific treatment with the tyrosinekinase inhibitor Imatinib (STI571) without any other previous regimen (Druker et al., 2001; Kantarjian et al., 2002; O'Brien et al., 2003; Kvasnicka et al., 2004b). In a second group of 125 patients with allogeneic BM transplantation (BMT) we analyzed a total of 516 samples that were taken at standardized intervals in the pre- and post-transplant period. The vascular structures (sinusoids, capillaries) in the BM were identified by immunohistochemistry with CD34 and CD105. Extending the normally applied hot spot method (Weidner et al., 1991), all specimens were evaluated by exact computer-assisted morphometric analysis at 500 x magnification. Assessment was performed by randomly selecting 50 fields of 3.77×10^{-2} mm^2 each (total of 1.884 mm^2 BM area per biopsy). Because of the increasing amount of adipose tissue, particularly following therapy and BMT (Thiele et al., 2001b), measurements of the total BM area may unduly reduce the calculated number of microvessels. For this reason, the areas occupied by fat cells were not regarded in our analysis and the frequency of microvessels or MVD was expressed per square millimeter (mm^2) of

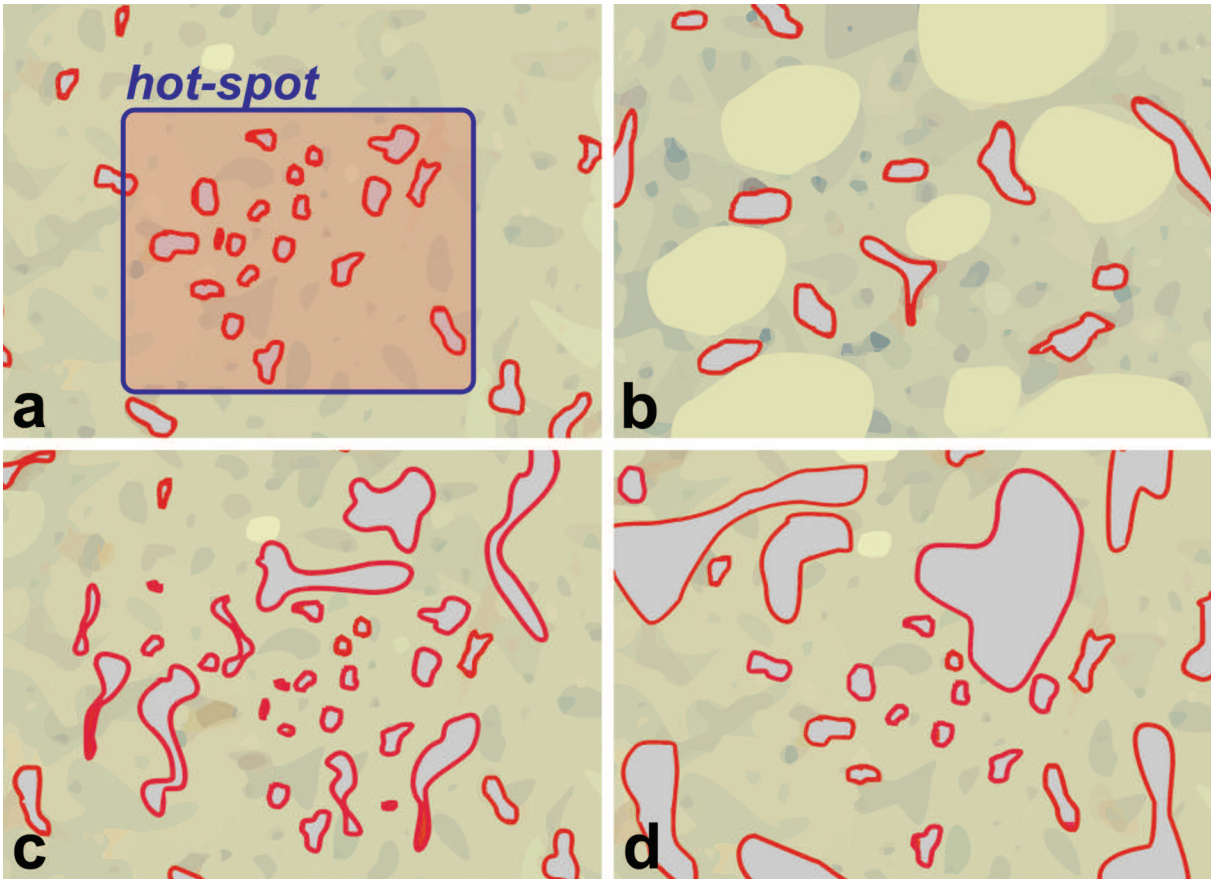


Fig. 2. Quantification of bone marrow vessel density in hematopoietic neoplasias: according to the so-called hot spot technique only areas with intense neovascularization are regarded **(a)**. Therefore in cases with increased amount of fat cells **(b)**, in particular after chemotherapy or transplantation, marrow vascularity is underestimated. Furthermore, functional characteristics of perfusion like tortuosity and branching of vessels **(c)** as well as vascular area - luminal distension **(d)** should be evaluated by elaborate morphometric analysis.

hematopoietic tissue. In addition, the microvessel area (MVA) indicative of luminal distension and several other morphologic parameters characterizing the tortuosity (irregularity of shape) and branching of the vascular structures were assessed (form factor, maximal length, deviation from circularity). In particular determination of MVA implies assessment of luminal width (Aguayo et al., 2003) that in association with the MVD has a significant impact on perfusion. The calculation of the latter features was especially important, since blood flow is physically different in straight versus tortuous and brached vessels (Kvasnicka and Thiele, 2002) and consequently allows a more advanced insight into functional aspects of the microvasculature (Korkolopoulou et al., 2001).

Compared with normal BM at diagnosis of CML a significant higher MVD can be observed (Table 2) and in line with this finding, a remarkable increase in the mean MVA is also found (Fig. 3a). The vessels are smaller, more irregularly shaped (Fig. 3b) and reveal a conspicuous tortuosity in CML specimens (Table 2). An increase in argyrophilic fibers was significantly associated with a higher MVD (Table 3). However, even

Table 2. Characteristics of angiogenesis at diagnosis of CML (n=85) compared to normal bone marrow (n=20).

	CML		CONTROL
	MEAN	95% CI	MEAN
Microvessel density (MVD) (per mm^2 hematopoiesis)	131*	120-142	76
Microvessel area (MVA) (μm^2)	130*	117-142	77
Tortuosity of microvessels (maximal length in µm)	7.7*	7.3-8.1	134
Form factor (deviation of shape)	0.46*	0.45-0.49	70

* CML vs. control: $p < 0.01$

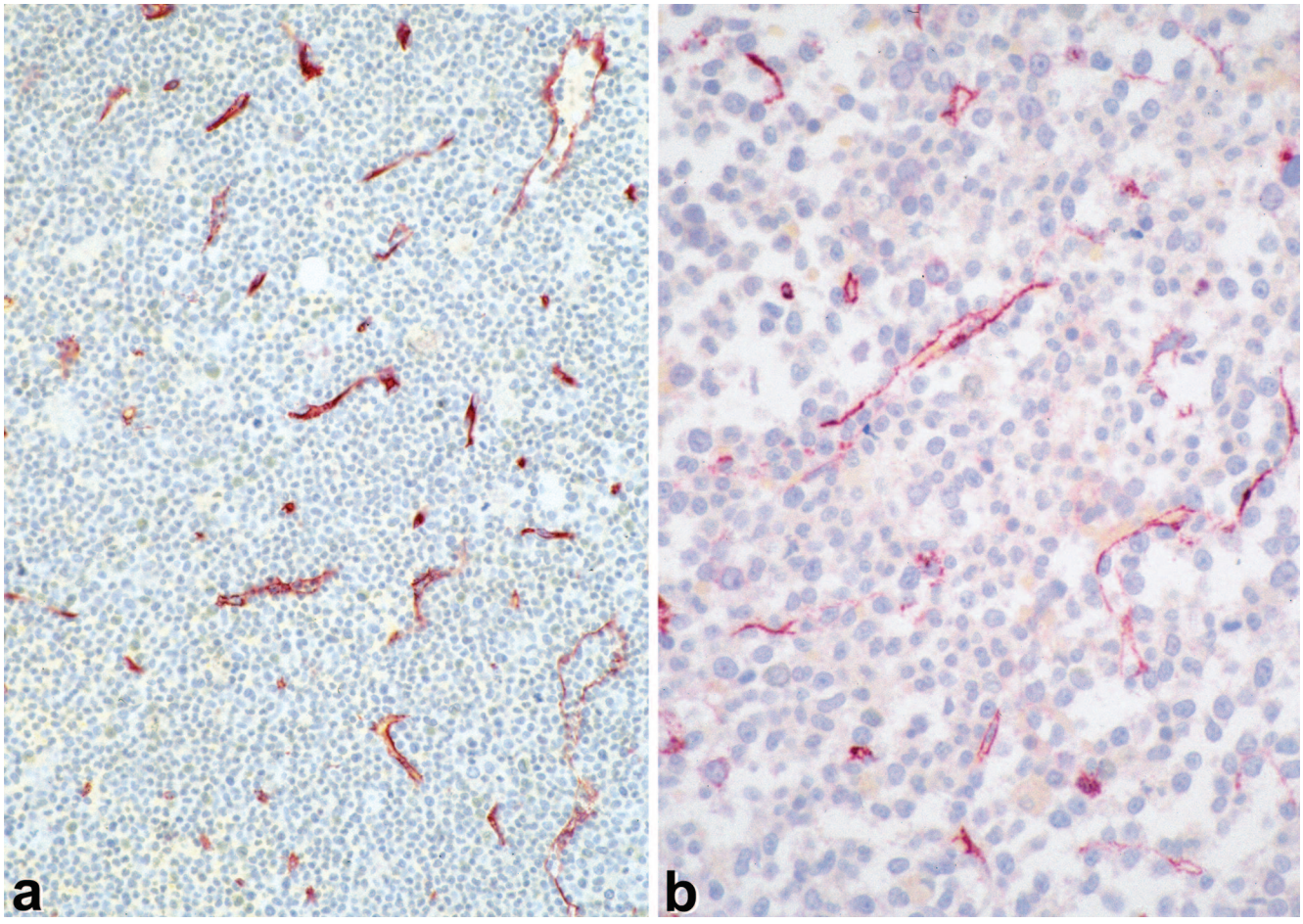


Fig. 3. Bone marrow angiogenesis at diagnosis of Ph^{1+} CML: the hypercellular bone marrow reveals a significant increase in small and irregularly shaped vessels **(a)**. These show also an abnormal branching **(b)** with variable luminal distension. a, b, CD34. a, x 180; b, x 380

nonfibrotic cases of CML displayed a significantly increased amount of microvessels in the BM than the control group. Regarding the impact of therapy, interestingly IFN failed to reduce the number of microvessels and was often accompanied by an increase in fibers (Fig. 4a-c). Only 37.5% of patients under IFN treatment revealed a significant reduction of their MVD, contrasting more than 70% in the group with Imatinib (STI571) therapy (Fig. 5a). Concerning MVA, IFN therapy was able to reduce the vessel area in about 59%, similar to Imatinib and HU, thus indicating no relevant changes in luminal distension in relation to therapeutic regimens applied (Fig. 5b). Following BMT (Fig. 6a,b), a significant increase in the MVD was found in the early post-transplant period (Table 4), but after 6 months a normalization of the MVD could be observed (Fig. 6c,d). However, even after 6 months the MVA was higher than normal and the vessel architecture (form factor and maximal length) remained grossly disturbed (Table 4). There is no significant impact on engraftment, but the few cases with leukemic relapse revealed a higher MVD in the early post-transplant period. Anomalies of microvascular architecture were readily demonstrable by performing a three-dimensional computer-assisted reconstruction on serial sections before and after BMT (Fig. 7). In the pretransplant biopsies a network of irregular shaped, tortous and branching vessels could be demonstrated. In the early post-transplant period (day 20) the number of microvessels was apparently increased. This finding was also accompanied by an increased luminal area. After day 60 the complexity of the vascular structures was

Table 3. Association of microvessel density (MVD) and extent of bone marrow fibrosis at time of diagnosis in CML.

GRADE OF FIBROSIS	MEAN	95% CI
no (0)	121	100-142
initial (+)	135	118-151
manifest (+++)	141*	125-156

* (0) vs. (+++): $p < 0.02$

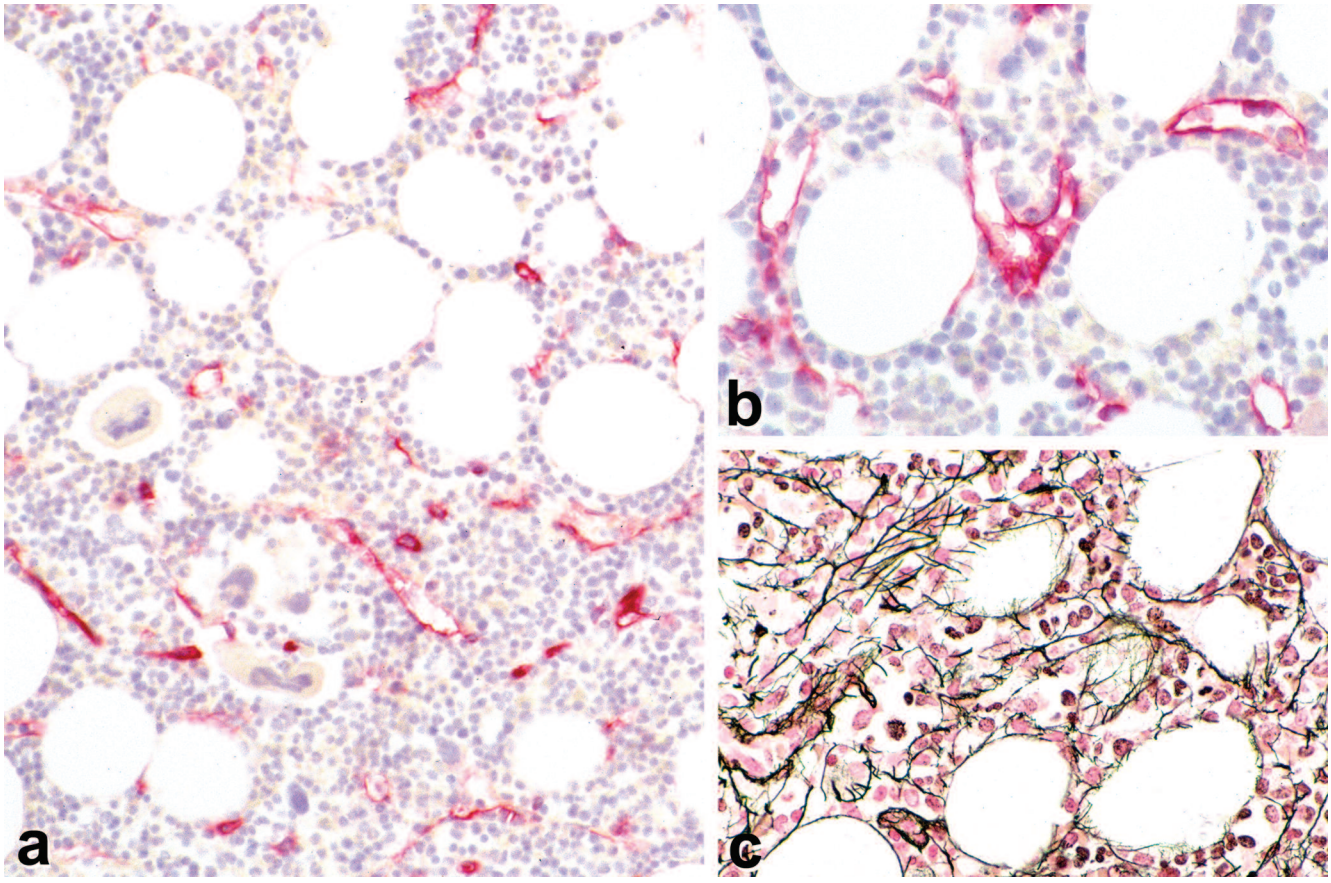


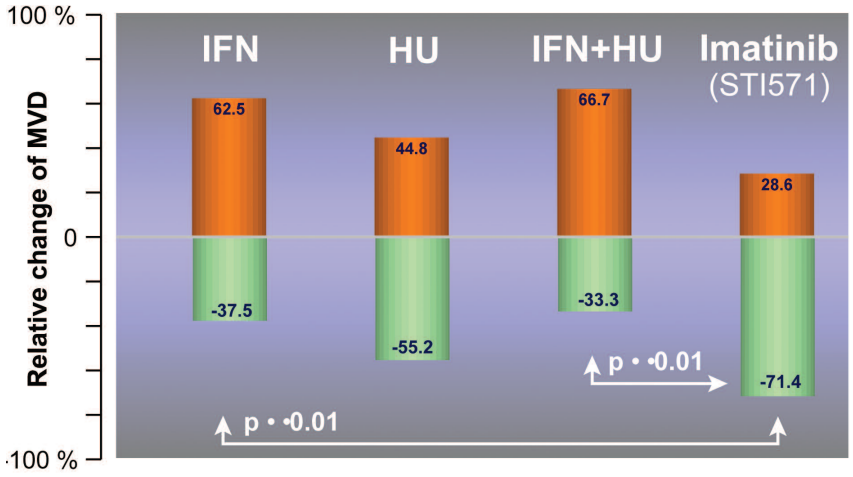
Fig. 4. Bone marrow angiogenesis in CML after treatment with IFN: although cellularity is reduced **(a)** treatment with IFN failed to reduce the number of marrow vessels **(b)**. In most patients this effect was associated with a further enhancement in marrow fibers **(c)**. a, b CD34; c, Silver impregnation. a, e x 180; b, x 380

reduced (Kumar et al., 2003), however, the vascular architecture was still disturbed showing iregular shaped and blind ending sinusoids (Fig. 7).

Altogether, our results underline the therapeutic potential of Imatinib (STI571), in particular its anti-angiogenic capacity (Rumpel et al., 2003). It has been

Table 4. Alterations of angiogenesis during bone marrow transplantation (BMT) in patients with CML (mean and range).

	BEFORE BMT (DAYS)		AFTER BMT (DAYS)	
	22±30	27±7	50±15	190±80
Microvessel density (MVD) (per mm^2 hematopoiesis)	79 (16-344)	121 (20-392)	93 (11-315)	71 (16-204)
Microvessel area (MVA) (μm^2)	211 (24-1,253)	291 (44-627)	258 (35-795)	172 (33-531)
Tortuosity of microvessels (maximal length in µm)	9.1 (3.5-18.0)	11.7 (4.8-17.4)	10.2 (4.8-22.8)	8.2 (4.1-15.4)
Form factor (deviation of shape)	0.55 (0.35-0.70)	0.56 (0.43-0.67)	0.56 (0.46-0.69)	0.57 (0.49-0.67)



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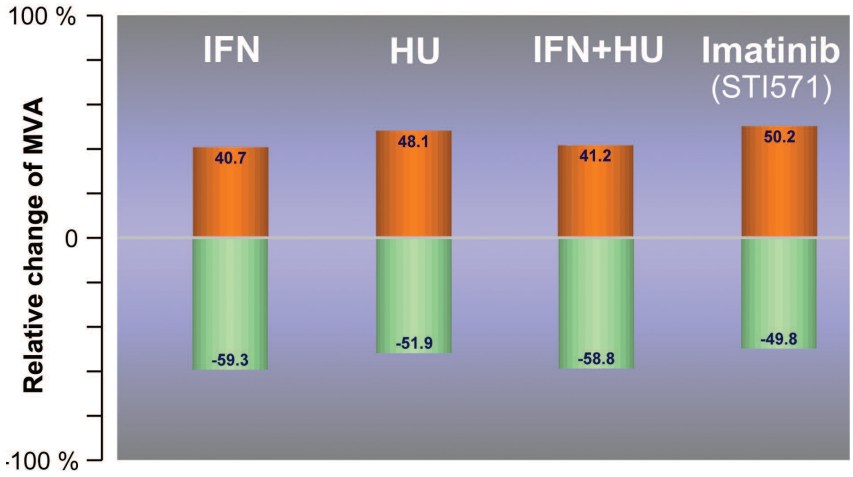

b

Fig. 5. Relative changes of microvessel density **(a)** and microvessel area **(b)** during therapy of Ph^{1+} CML.

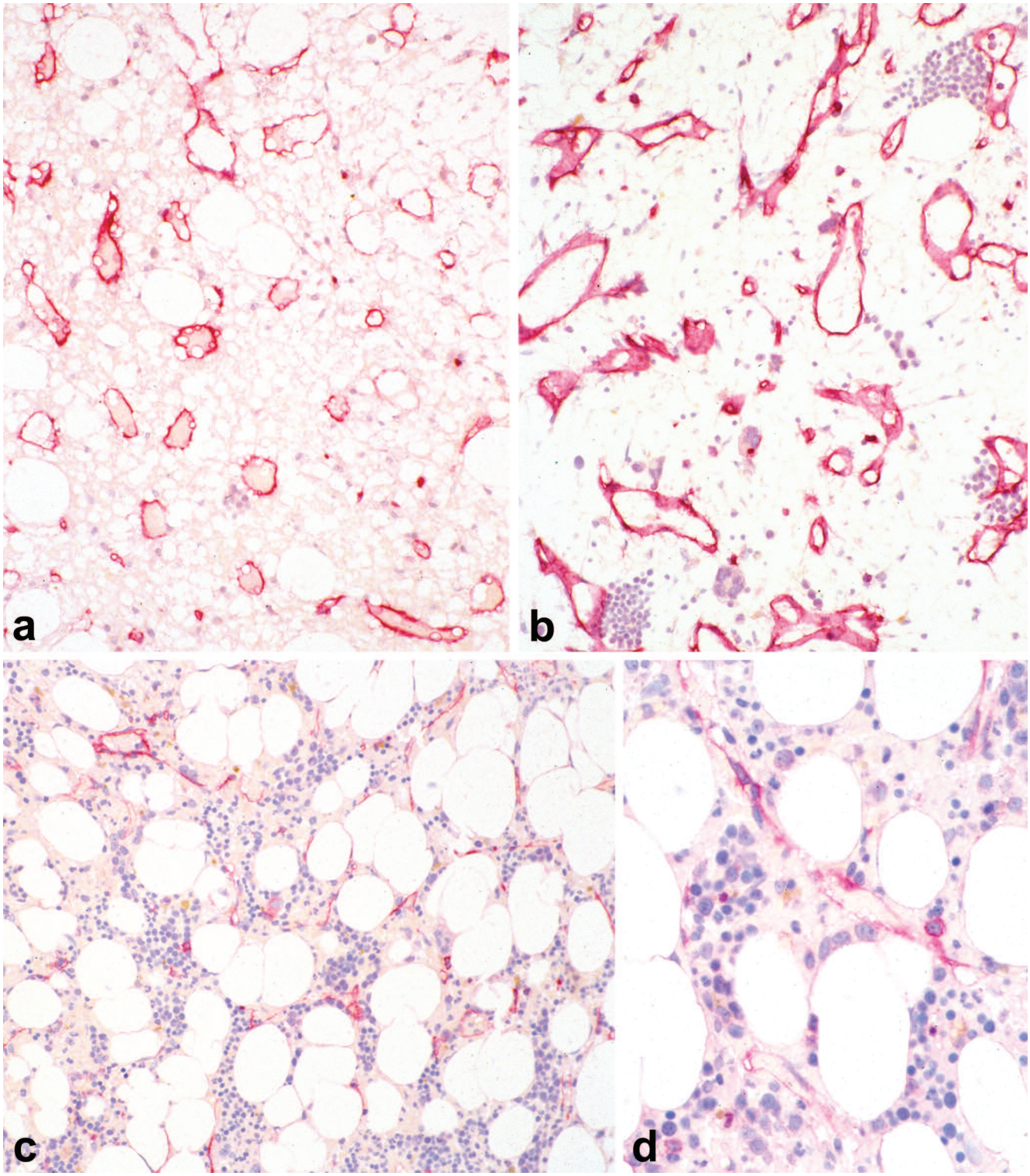

Fig. 6. Bone marrow angiogenesis in Ph^{1+} CML after BMT. **(a)** In the early post-transplant period (day 10) a large number of dilated sinus can be observed. **(b)** At day 20 this feature is accompanied by regenerating hematopoiesis, but the vessel lumina are still remarkably dilated. **(c, d)** Following successful engraftment at day 100 frequency of marrow vessels achieve normal values, however, the architecture of microvessels remains disturbed **(d)**. CD34. a, b, c x 180; d x 380

convincingly demonstrated that Imatinib reduces the BCR/ABL-mediated secretion of VEGF which is mainly responsible for the angiogenic effects of this drug (Ebos et al., 2002). The significant correlation between number of VEGF-positive BM cells and MVD (Lundberg et al., 2000) was supported by a corresponding decline of VEGF plasma levels in patients with decreased vascularity. Furthermore, in most patients cytogenetic response was also associated with a reduction of BM vascularity in the Imatinib group. On the other side, it might be concluded that IFN has only a limited anti-angiogenic effect in CML patients (Kvasnicka et al., 2004a,b). Interestingly, despite the putative angiogenic effect of IFN (Aguayo et al., 2000), involution of MVD lagged behind the reduction of the neoplastic population. Comparable observations were made by other groups that investigated different hematological disorders (Pruneri et al., 2003). On the other hand, recent data

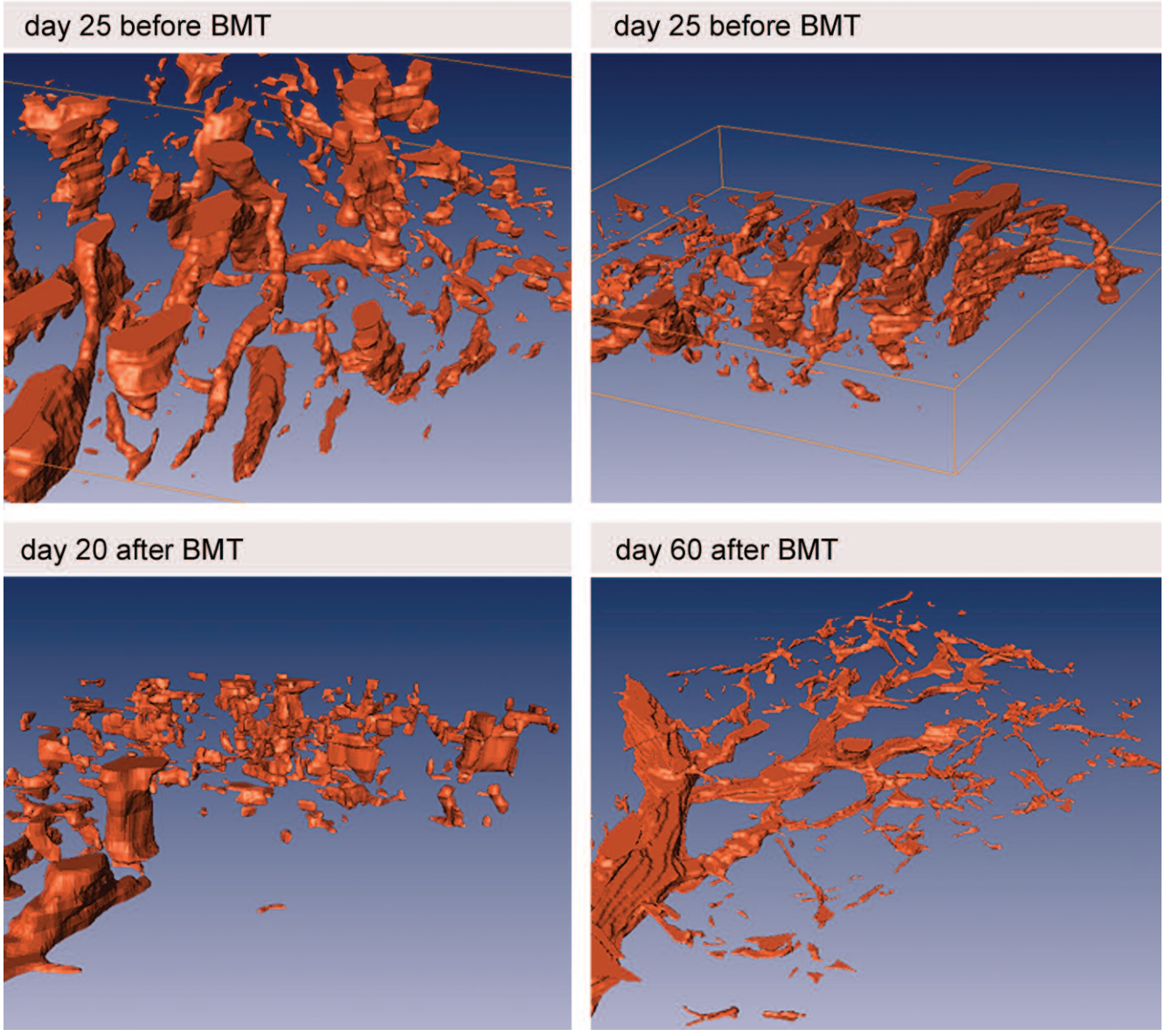

Fig. 7. Three-dimensional reconstruction of bone marrow angiogenesis following BMT: after chemotherapy of Ph^{1+} CML (day 25 before BMT) a complex network of branching and tortuous microvessels with blind ending sinuses can be observed. In the post-transplant period (day 20) the microvessels are remarkably dilated. Following successful engraftment and reconstitution of hematopoiesis at day 60 the complexity of the vascular structures is less conspicuous including a significantly reduced number of microvessels. However, the normal architecture still remains disturbed showing dilated and irregularly shaped sinusoids. Original magnification x 180

obtained from cultured human endothelial cells produced evidence that HU down-regulates endothelial gene expression (Brun et al., 2003). This pathomechanism might be responsible for the reduced MVD observed in the HU-treated cohort. Since Imatinib also targets PDGF receptor activity (Buchdunger et al., 2002), normalization of vascular structures may be also influenced by effects exerted on megakaryopoiesis (Beham-Schmid et al., 2002) by neutralizing its stimulating function on the BM stroma (Le Bousse-Kerdiles and Martyre, 1999b). However, increased angiogenesis is only one aspect of the complex alterations in cell physiology that is responsible for the malignant growth (Moehler et al., 2001). Therefore, it is possible that the specific efficacy of these drugs is also dependent on some other pathomechanisms of action that are probably as important as the anti-angiogenic potency. Thus, a further insight into the biology of CMPDs is an essential step for the development of new agents. In this context, it should be kept in mind that in hematological diseases it is very likely that the various cytokines mediating angiogenesis (VEGF, bFGF) are involved not only in an autocrine loop for hematopoietic malignant cells, but also in paracrine pathways (Fig. 1), where stromal cells, stimulated by malignant cells, produce growth factors for endothelial cells and the latter in turn release mediators for the neoplastic cell population (Fiedler et al., 1997). Therefore, three main factors are present in this complex scenario of increased angiogenesis: hematopoietic malignant cells, stromal cells and endothelial cells. It is likely that the inhibition of one of these different populations is not sufficient to eliminate the disease and in future more innovative therapeutic approaches should take into account the necessity to target all three components (Dickson and Shami, 2001; Stasi and Amadori, 2002).

Other subtypes of CMPDs

Contrasting Ph^{1+} CML, the Ph^{1-} subtypes of CMPDs that predominantly include chronic idiopathic myelofibrosis (IMF), polycythemia vera (PV) and essential thrombocythemia (ET) have been relatively rarely studied concerning impact and dynamics of angiogenesis. Only advanced stages of IMF and also ill-defined subtypes with secondary myelofibrosis characterized by myeloid metaplasia and collagen fibrosis in the BM were regarded in larger samples of patients (Lundberg et al., 2000; Mesa et al., 2000). In comparison to controls in these patients a higher grade of angiogenesis was found in 70% contrasting 33% of patients with PV and 12% of cases with ET (Mesa et al., 2000). Previous studies were in keeping with an enhanced deposit of subendothelial collagen type IV in full blown IMF (Reilly et al., 1985; Apaja-Sarkkinen et al., 1986; Lisse et al., 1991; Thiele et al., 1992) and an increase in vascularity associated with advanced myelofibrosis. These features together with an increased dilatation and tortuosity of vessels (Thiele et al., 1992) were held responsible for the higher perfusion rate recognized in this disorder (Van Dyke et al., 1971; Charbord, 1986). Three-dimensional reconstitution (Lundberg et al., 2000) as well as determination of certain morphometric factors like tortuosity, width of vascular lumina and maximal length of vessels (Table 5) were in keeping with these findings (Fig. 8a-c). Furthermore, changes in the vascular structures are usually accompanied by a conspicuous intraluminal hematopoiesis (Georgii et al., 1998; Thiele et al., 1989, 1994, 2001a). This feature (Fig. 8c) has been assumed to present the underlying pathomechanism for the occurrence of the leuko-erythroblastic blood picture and myeloid metaplasia characterizing these disorders (Reilly et al., 1985; Wolf and Neiman, 1985; Lisse et al., 1991). For this reason, a multivariate analysis of the increased angiogenesis in IMF was significantly associated with increased spleen size and also considered as an independent risk factor for overall survival (Mesa et al., 2000).

However, the data on IMF has to be significantly modified when regarding the new WHO-classification (Thiele et al., 2001a) that recognizes also the very early

Table 5. Characteristic features of angiogenesis in Ph^{1-} CMPDs (mean and 95% CI).

	IMF (n= 85)	PV (n=90)	ET (n=20)	CONTROL
Microvessel density (MVD) (per mm^2 hematopoiesis)	120 (110-129)	113 (104-123)	104 (85-123)	76
Microvessel area (MVA) (μm^2)	195 (171-217)	371 (320-420)	127 (113-141)	77
Tortuosity of microvessels (maximal length in µm)	22 (20-23)	31 (29-33)	24 22-26)	134
Form factor (deviation of shape)	0.66 (0.65-0.67)	0.61 (0.59-0.62)	0.54 (0.52-0.56)	70

Table 6. Differences of angiogenesis (mean and 95% CI) in patients with IMF according to stage (advance of myelofibrosis/osteosclerosis).

	PREFIBROTIC/ EARLY STAGE (n=40)	MANIFEST/ ADVANCED STAGE (n=45)
Microvessel density (MVD) (per mm^2 hematopoiesis)	98[a] (86-111)	143 (133-152)
Microvessel area (MVA) (μm^2)	172[b] (138-206)	(188-249)
Tortuosity of microvessels (maximal length in µm)	20 (18-22)	22 (21-24)
Form factor (deviation of shape)	0.65 (0.64-0.67)	0.67 (0.65-0.68)

[a]: MVD early vs. advanced stage $p < 0.02$; [b]: MVA early vs. advanced stage $p < 0.05$

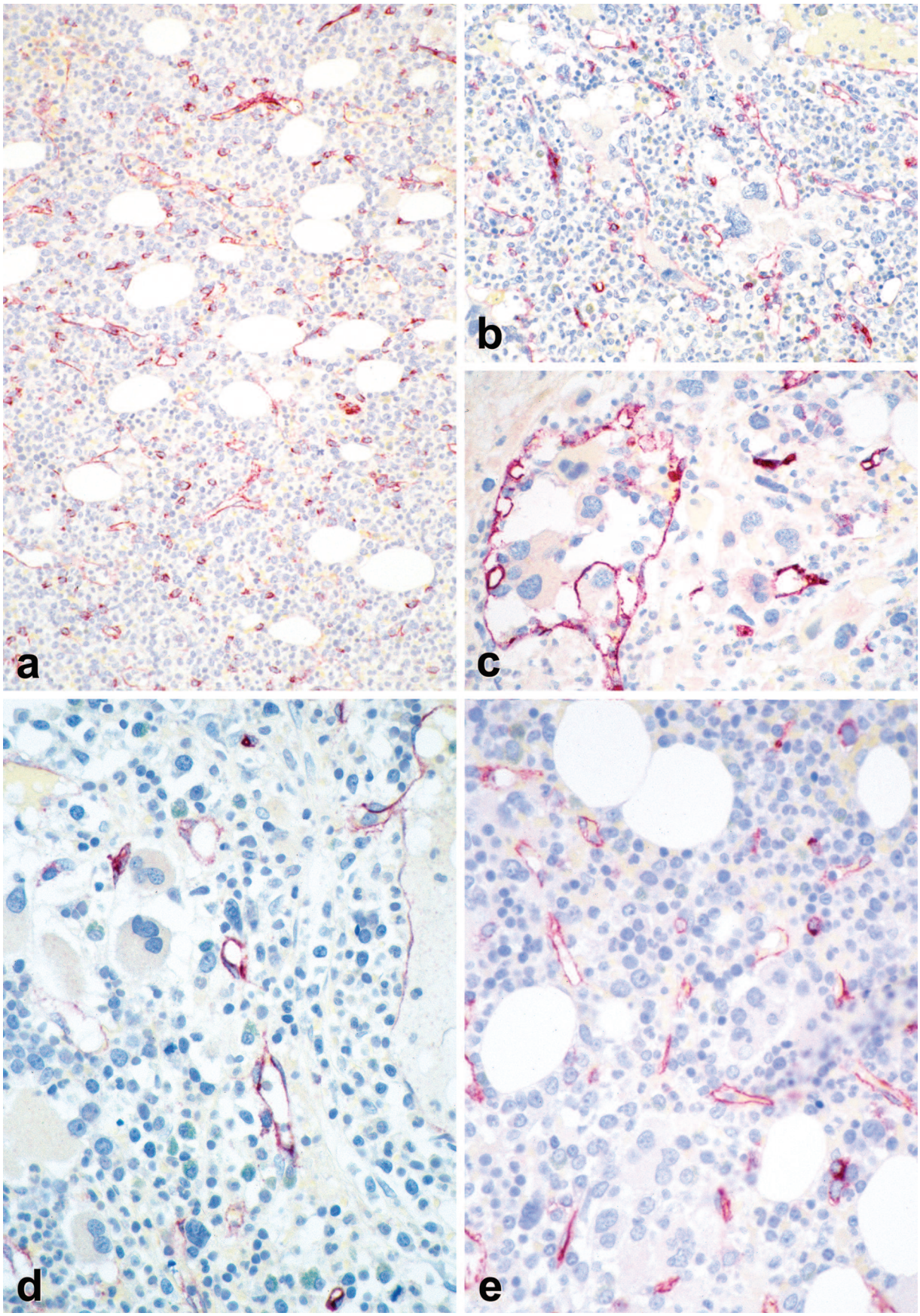

Fig. 8. Bone marrow angiogenesis in Ph^{1-} subtypes of CMPDs: in early stages of IMF **(a)** no significant increase in the frequency of microvessels is observed. Advanced stages of the disease **(b, c)** are characterized by an enhancement of microvessel density which is accompanied by a remarkable distension of vascular lumina. In these cases an intrasinusoidal hematopoiesis is a frequent finding **(c)**. Contrasting PV **(d)** in ET **(e)** no overt increase in the number of microvessels is recognizable. CD34. a, x 180; b-e, x 380

stages of this stepwise evolving disease process. As has been emphasized, the prefibrotic and early fibrotic precursor lesions do not exhibit an increased vascularity or intraluminal hematopoiesis (Kvasnicka and Thiele, 2002; Thiele et al., 1992) thus contrasting the more advanced fibrosclerotic (manifest) stages (Table 6). Former data indicating no relationship between angiogenesis and reticulin fibrosis in IMF have to be further discussed with reference to the obvious bias of selection of patients (Mesa et al., 2000). The latter series obviously also included patients with post-polycythemic and post-thrombocythemic myelofibrosis presenting with overt leuko-erythroblastosis, splenomegaly and dacryocytosis, i.e. advanced stages that do not fully conform with the WHO classification of IMF (Thiele et al., 2001a). In this context, it has been amply demonstrated that release of a number of cytokines (bFGF, TGF-ß, PDGF) from megakaryocytes and makrophages plays a crucial role not only for the development of myelofibrosis, but also for the induction of angiogenesis (Donovan et al., 1997; Berse et al., 1999; Le Bousse-Kerdiles and Martyre, 1999a,b). In this complex concert of various cell to cell interactions VEGF and bFGF are also prominently involved (Ferrara, 1996; Mandriota and Pepper, 1997; Salcedo et al., 1999). Accordingly few studies were able to demonstrate increased VEGF-positive BM cells compared with controls (Lundberg et al., 2000) or an elevated serum level for this angiogenic factor (Di Raimondo et al., 2001). Moreover, *in vitro* isolated megakaryocytes derived from CMPDs were shown to exhibit an increased bFGF expression (Bock et al., 2002). Altogether, this complex functional network involves angiogenesis and myelofibrosis in the BM of Ph^{1+} and Ph^{1-} subtypes of CMPDs and therefore is in keeping with our result of a close mutual relationship as shown in Tables 3 and 6.

It should not be overlooked that angiogenesis in IMF presents a therapeutic target with the prospect to inhibit neo-angiogenesis associated with the fatal myelofibrotic BM changes. In this context, a conflict of opinion is evident concerning the administration of thalidomide in these patients (D'Amato et al., 1994; Kenyon et al., 1997; Thomas and Kantarjian, 2000), in particular since a favorable effect has been observed in multiple myeloma (Rajkumar and Witzig, 2000) and myelodysplastic syndromes (Strupp et al., 2002; Zorat and Pozzato, 2002). First studies with this drug produced controversial results reagarding its safety and efficacy (Mangi and Newland, 2000; Barosi et al., 2001; Canepa et al., 2001; Elliott et al., 2002; Piccaluga et al., 2002; Bonn, 2003). Another agent that may exert an inhibitory effect on angiogenesis is Imatinib (STI571) that has been recently and successfully applied in CML patients (Druker et al., 2001; Kantarjian et al., 2002; Kvasnicka et al., 2004b). This tyrosine kinase inhibitor also interferes with the activity of cytokines which are involved in angiogenesis (Buchdunger et al., 2000) and therefore a positive response was postulated when treating patients with IMF. Although preliminary data are in keeping with an improvement, severe complications (Hasselbalch, 2001; Tefferi et al., 2002) prevent a further application as monotherapy, unless it may be included in combinations with conventional cytoreductive regimens (Bauer et al.,2003).

In comparison with IMF (Fig. 8a-c) only little information is available concerning angiogenesis in PV (Fig. 8d) and ET (Fig. 8e). In PV a striking distension of vascular structures due to intraluminal erythrocyte sludging was noted (Fig. 8d), but not a significant increase in vessels characterized by a basement membrane with ensuing anti-collagen type IV staining reaction (Thiele et al., 1992). Contrasting this previous finding by applying CD34 and CD105 immunohisto-chemistry the smaller sinusoids were additionally demonstrable and definitely revealed an overall increased vascularity associated with a prominent branching and luminal distension (Lundberg et al., 2000; Mesa et al., 2000; Kvasnicka and Thiele, 2002). This result emphasizes again a critical evaluation of special techniques when trying to determine angiogenesis in the BM (Table 1). Similar to the prodromal prefibrotic or early fibrotic stages of IMF (Table 6), available data concerning ET significantly depends on the applied diagnostic criteria. By using the WHO classification (Imbert et al., 2001) a more stringend approach to the problem of differentiation between the various subtypes of CMPDs presenting with an excess in platelets is certainly accomplished (Thiele and Kvasnicka, 2003b). Following explicitly these diagnostic guidelines, ET reveals a mild increase in the number of vessels (Fig. 8e), but not a severe deviation from normal vascular structures as may be derived from the findings detailed in Table 5. On the other hand, data from the current literature that is based on diagnostic criteria established by the PVSG (Murphy et al., 1997; Murphy, 1999) show an increased angiogenesis associated with splenomegaly and a higher risk for the development of myeloid metaplasia (Mesa et al., 2000, 2002) combined with an increased serum concentration of VEGF (Di Raimondo et al., 2001). In particular regarding the data on spleen size and myeloid metaplasia it may be speculated that based on the rather crude diagnostic criteria applied in these patients, a fraction of cases may actually present with early stage IMF but not (true) ET (Thiele and Kvasnicka, 2003a,b).

In conclusion, the usually applied so-called hot spot technique of assessing angiogenesis should be replaced by more elaborate methods (i.e. morphometric analysis) to provide a more advanced insight into quantity and especially quality of vascular structures. In CMPDs angiogenesis is significantly associated with evolution of myelofibrosis and may be altered by certain therapeutic regimens interfering with cytokine release and various cell to cell interactions like IFN, HU and Imatinib (STI571) in CML.

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