Increased Angiogenesis in the Bone Marrow of Patients with Systemic Mastocytosis

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Recent data suggest that angiogenesis in the bone marrow (BM) is augmented and associated with growth of neoplastic cells in various hematological malignancies. Systemic mastocytosis (SM) is a neoplasm affecting multilineage and mast cell (MC)-committed hemopoietic progenitors. In the present study, we have assessed the BM microvessel density (MVD) by CD34 immunohistochemistry in 21 patients with SM, 5 with cutaneous mastocytosis (no BM infiltrates), and 5 control cases (normal BM). The median BM MVD was significantly higher in SM compared to cutaneous mastocytosis or controls ($P < 0.05$). In addition, a significant correlation ($r = 0.74$) between the BM MVD and grade of MC infiltration (percent tryptase⁺ BM infiltrates) was found in SM. Moreover, the MVD was higher in MC infiltrates compared to the nonaffected adjacent marrow ($P < 0.05$). Immunohistochemical staining revealed expression of vascular endothelial growth factor in MC infiltrates. The notion that SM is associated with increased BM angiogenesis and vascular endothelial growth factor expression may have implications for the biology of disease and development of new treatment strategies. (*Am J Pathol* 2002, 160:1639–1645)

Mastocytosis is a term used for a heterogeneous group of disorders characterized by abnormal growth and accumulation of mast cells (MCs) in one or more organs.^{1–4} Cutaneous mastocytosis (CM) is commonly diagnosed in childhood and has a favorable prognosis.⁵ In a high percentage of cases with pediatric CM, spontaneous regression is found. Systemic mastocytosis (SM) can develop at any age and is characterized by multiorgan involvement and disease persistence.^{1–4} In most SM patients, somatic mutations in the kinase domain of *c-kit* are detectable.^{6–8}

The majority of patients with SM show an indolent clinical course throughout decades.^{1–4} However, in a smaller subset, progression to aggressive or even leukemic disease is seen.^{1–4,9–12} These patients acquire organopathy caused by progressive MC infiltrates in diverse organs such as the bone marrow (BM), liver, or skeletal system.^{9–14} The factors that contribute to the progression of SM, however, have not yet been defined.

Recent data suggest that angiogenesis contributes to the viability and growth of neoplastic cells in various malignancies.^{15–17} The angiogenic growth factors involved are produced by tumor cells or accessory cells.^{16,17} One of the most important angiogenic growth factors involved in tumor angiogenesis seems to be vascular endothelial growth factor (VEGF).^{16,17}

So far, angiogenesis has mainly been described for solid tumors.^{16–18} More recently, however, increased neoangiogenesis has also been described for various hematological malignancies such as acute or chronic myeloid leukemias.^{19–22} The aims of the present study were to measure BM angiogenesis in patients with SM and to correlate the microvessel density (MVD) with well-established markers of disease.

Patients and Methods

Patients and Staging Investigations

Twenty-one patients with SM, 5 with CM [subtype, urticaria pigmentosa (UP)], and 5 control cases (no hematological neoplasm) were examined. Mastocytosis was diagnosed according to established criteria.²³ In the SM group, 17 patients had indolent SM, 3 slowly progressing, smoldering SM, and 1 aggressive SM. Patients with an associated clonal hematological non-MC lineage disease were excluded. Staging investigations included physical examination, ultrasound (spleen), X-ray of bones, BM examination, blood picture and chemistry, and measurement of total (α/β) tryptase levels by fluoroenzyme immunoassay (Pharmacia, Uppsala, Sweden). Histamine levels were measured by commercial radioimmunoassay

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Table 1. Patients' Characteristics, Clinical Findings, and Disease-Related Symptoms: Comparison with BM MVD

No.	Diagnosis	Sex	Age at onset of disease	UP-like skin lesions	Flush	Vascular instability	GI-ulcer	Palpable spleno-megaly	Osteolysis	Mean MVD MV/field	Mean MVD in infiltrate MV/field
01	ASM	m	22	No	No	Yes	Yes	No	Yes	13.3	35.8
02	ISM	f	30	Yes	Yes	Yes	No	No	No	07.1	23.6
03	ISM	f	26	Yes	No	No	No	No	No	10.4	19.2
04	ISM	f	36	Yes	No	No	No	No	No	43.4	55.2
05	ISM	m	20	Yes	No	No	No	No	No	11.8	25.8
06	ISM	m	33	Yes	No	No	No	No	No	21.2	29.2
07	ISM	f	32	Yes	No	No	No	No	No	12.3	33.6
08	ISM	m	48	Yes	No	No	n.k.	No	n.k.	01.5	08.3
09	ISM	f	33	Yes	No	No	No	No	No	07.9	16.4
10	ISM	f	13	Yes	No	No	No	No	No	13.7	38.2
11	ISM	f	20	Yes	No	No	No	No	No	12.4	18.8
12	ISM	f	21	Yes	Yes	No	Yes	No	No	20.6	45.0
13	ISM	m	44	Yes	Yes	Yes	No	No	No	19.4	42.0
14	ISM	m	28	Yes	No	No	No	No	n.k.	10.5	28.8
15	ISM	m	61	Yes	Yes	No	No	Yes	No	07.0	24.3
16	ISM	m	54	Yes	No	Yes	No	No	No	01.9	07.0
17	ISM	f	27	Yes	Yes	Yes	Yes	No	No	32.6	92.4
18	ISM	f	50	Yes	No	No	n.k.	No	No	02.5	08.7
19	SSM	f	01	Yes	No	No	No	Yes	n.k.	23.7	36.9
20	SSM	f	21	Yes	Yes	Yes	No	Yes	No	26.1	43.2
21	SSM	m	20	Yes	No	Yes	No	No	No	30.9	68.7

ASM, aggressive systemic mastocytosis; ISM, indolent systemic mastocytosis; SSM, smoldering systemic mastocytosis; UP, urticaria pigmentosa; GI, gastrointestinal; MV, microvessel; n.k., not known; m, male; f, female.

(Immunotech, Marseille, France). Informed consent was obtained in each case. The patients' characteristics and clinical findings are shown in Table 1. Laboratory parameters are depicted in Tables 2 and 3.

Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded, formalin-fixed BM sections using the indirect immunoperoxidase staining technique.²⁴ Endogenous

peroxidase was blocked by methanol/H₂O₂. The anti-tryptase monoclonal antibody (mAb) G3 (work dilution, 1:5000) was purchased from Chemicon (Temecula, CA), the CD34 mAb QBEND 10 (1:100) from Immunotech, and a rabbit anti-VEGF antibody (work dilution, 1:50) as well as a blocking (VEGF-based) peptide from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies were diluted in 0.05 mol/L of Tris-buffered saline (pH 7.5) plus 1% bovine serum albumin and applied for 60 minutes. In the case of VEGF staining, sections were pretreated by mi-

Table 2. Bone Marrow Findings and Hematologic Parameters: Comparison with MVD

No.	Diagnosis	% MC in BM section	% MC in BM smears	WBC, ×10 ⁹ /L	ANC, ×10 ⁹ /L	Eo, %	Ba, %	Hb, g/dL	plt, 10 ⁹ /L	Mean MVD MV/field	Mean MVD in infiltrate MV/field
01	ASM	05	1	4.3	1.2	1	0	12.2	208	13.3	35.8
02	ISM	20	1	7.4	5.4	1	0	13.2	269	07.1	23.6
03	ISM	10	1	8.9	6.8	0	0	13.6	301	10.4	19.2
04	ISM	65	2.5	4.9	2.8	0	0	13.1	280	43.4	55.2
05	ISM	15	1	8.4	5.9	2	0	15.6	341	11.8	25.8
06	ISM	25	1	6.0	3.7	3	0	13.8	220	21.2	29.2
07	ISM	15	<1	7.4	4.7	1	0	12.7	272	12.3	33.6
08	ISM	05	<1	7.0	4.0	2	1	14.0	197	01.5	08.3
09	ISM	10	<1	6.2	3.7	0	1	13.2	174	07.9	16.4
10	ISM	30	1.5	7.0	4.9	0	0	12.5	320	13.7	38.2
11	ISM	01	<1	6.6	3.2	3	0	13.6	256	12.4	18.8
12	ISM	05	1	5.1	3.1	2	0	12.4	205	20.6	45.0
13	ISM	20	<1	7.3	5.4	0	0	14.4	231	19.4	42.0
14	ISM	05	2.5	5.5	2.8	1	0	14.3	265	10.5	28.8
15	ISM	02	<1	6.2	3.9	2	1	14.8	128	07.0	24.3
16	ISM	15	2	5.4	3.5	3	1	14.9	238	01.9	07.0
17	ISM	30	<1	3.8	2.0	1	0	14.9	215	32.6	92.4
18	ISM	20	n.d.	5.3	3.3	3	0	13.7	154	02.5	08.7
19	SSM	25	1	30.1	21.7	6	0	13.5	405	23.7	36.9
20	SSM	70	4	5.3	3.5	0	0	11.2	212	26.1	43.2
21	SSM	70	<1	4.7	3.7	0	0	14.7	207	30.9	68.7

ASM, aggressive systemic mastocytosis; ISM, indolent systemic mastocytosis; SSM, smoldering systemic mastocytosis; n.d., not done; WBC, white blood cell count; ANC, absolute neutrophil count; Eo, eosinophils; Ba, basophils; Hb, hemoglobin; plt, platelets; MV, microvessel.

Table 3. Laboratory Findings and Blood Levels of MC Mediators: Comparison with BM MVD

No.	Diagnosis	LDH, U/L	Fgen, mg/dl	aP, U/L	WB-T, ng/ml	S-T, ng/ml	WB-H, ng/ml	S-H, ng/ml	Mean MVD MV/field	Mean MVD in infiltrate MV/field
01	ASM	177	349	270	41.3	72.0	71.1	1.4	13.3	35.8
02	ISM	125	326	154	366.0	790.0	16.3	2.3	07.1	23.6
03	ISM	129	n.d.	99	35.2	47.9	32.6	1.6	10.4	19.2
04	ISM	81	344	188	172.2	352.5	13.7	3.6	43.4	55.2
05	ISM	n.d.	323	118	23.0	34.4	51.1	1.3	11.8	25.8
06	ISM	92	245	162	90.6	149.0	26.7	3.6	21.2	29.2
07	ISM	131	449	143	34.6	69.7	34.0	2.3	12.3	33.6
08	ISM	n.d.	329	128	n.d.	n.d.	n.d.	n.d.	01.5	08.3
09	ISM	n.d.	265	133	n.d.	22.1	n.d.	n.d.	07.9	16.4
10	ISM	109	276	128	56.8	113.0	n.d.	n.d.	13.7	38.2
11	ISM	133	235	58	15.5	16.5	80.9	1.05	12.4	18.8
12	ISM	127	230	74	27.6	53.7	44.8	0.89	20.6	45.0
13	ISM	113	322	86	79.6	108.0	116.0	2.20	19.4	42.0
14	ISM	152	211	109	8.6	16.7	29.4	1.11	10.5	28.8
15	ISM	n.d.	262	79	n.d.	28.2	n.d.	n.d.	07.0	24.3
16	ISM	133	380	97	27.0	47.5	44.2	0.22	01.9	07.0
17	ISM	187	318	122	90.6	175.0	22.5	1.07	32.6	92.4
18	ISM	135	n.d.	93	n.d.	n.d.	n.d.	n.d.	02.5	08.7
19	SSM	157	506	161	76.0	148.0	171.0	n.d.	23.7	36.9
20	SSM	104	290	82	748.0	1090.0	33.8	5.19	26.1	43.2
21	SSM	83	365	146	465.0	970.0	11.5	1.05	30.9	68.7

ASM, aggressive systemic mastocytosis; ISM, indolent systemic mastocytosis; SSM, smoldering systemic mastocytosis; n.d., not done; LDH, lactate dehydrogenase; Fgen, fibrinogen; WB-T, whole blood tryptase; S-T, serum tryptase; WB-H, whole blood histamine; S-H, serum histamine.

crowave oven. In selected experiments, the anti-VEGF antibody was preincubated with a specific blocking peptide before staining. After washing, slides were incubated with biotinylated horse anti-mouse or biotinylated goat anti-rabbit IgG for 30 minutes, washed, and exposed to avidin-biotin-peroxidase or streptavidin-biotin-peroxidase complex for 30 minutes. 3-amino-9-ethyl-carbozole (AEC) was used as chromogen. Slides were counterstained in Mayer's hemalaun. MC infiltrates were made visible by tryptase staining. Staining reactions obtained with antibodies against CD34, VEGF, and tryptase were examined on adjacent sections.

Determination of MVD

The MVD was determined in CD34-stained BM sections essentially as described by Perez-Atayde and colleagues.²⁵ In a first step, the entire marrow space of the sections (at least 20 microscopic fields) was examined. In a second phase, MC infiltrates (five fields per case) and the nonaffected adjacent BM (five fields per case) were examined separately. Microvessels were defined by their typical histological appearance and reactivity with CD34 mAb. The MVD was expressed as the mean number of microvessels per field.

Statistical Evaluation

The level of significance was determined by standard statistical tests including Kruskal-Wallis and Mann-Whitney *U* test. To determine correlations between MVD and other disease-related parameters [UP-like skin lesions, flush, vascular instability, gastrointestinal ulcerative disease, BM infiltration grade (tryptase⁺ MC), percent MCs

in BM smears, absolute neutrophil count, percent blood eosinophils, percent blood basophils, lactate dehydrogenase, fibrinogen, alkaline phosphatase, whole blood, serum tryptase levels, and histamine levels] linear correlations were applied. In case of multiple comparisons, the *P* values were adjusted according to Bonferroni.

Results

Evaluation of MVD in SM, CM (UP), and Controls

The BM MVD (microvessels per field) was significantly higher in patients with SM compared to CM/UP or controls [median BM MVD in SM: 12.4 (range: 1.5 to 43.4) *versus* UP median: 1.8 (range: 0.2 to 5.0) *versus* control median: 3.0 (range: 1.4 to 4.3), *P* < 0.05] (Figure 1). As assessed by serial section staining using a CD34 mAb and an anti-tryptase mAb, the increase in the BM MVD was found to result from a high vessel density in the MC infiltrates. Thus, when analyzed by morphometry, the median MVD in the SM infiltrates was significantly higher compared to the nonaffected adjacent marrow [SM infiltrates: 29.2 (7.0 to 92.4) *versus* nonaffected BM: 5.2 (1.0 to 15.0), *P* < 0.05] (Figure 2). Figure 3 shows examples of expression of tryptase (Figure 3A) and CD34 (Figure 3, B and C) in MC infiltrates in SM. Serial section staining confirmed that most of the CD34⁺ microvessels (Figure 3B) were located in the tryptase⁺ MC infiltrates (Figure 3A).

Detection of VEGF in MC Infiltrates

Using an antibody against VEGF, we were able to show that the MCs in BM MC infiltrates in SM express VEGF-

Figure 1. Determination of BM MVD. The MVD was determined in SM, CM, and normal BM (co). The figure shows the MVD values for each individual donor as well as the median MVD values in each group (horizontal bars).

immunoreactive material in all cases examined (SM, $n = 6$) (Figure 3D). A number of other cells in the BM including megakaryocytes and some immature myeloid progenitor cells were also labeled. By contrast, erythroid progenitor cells and most lymphoid cells did not express detectable VEGF (Table 4). The staining reactions obtained with the anti-VEGF antibody in the BM sections analyzed were completely abolished by preincubation with a VEGF-based blocking-peptide, confirming the specificity of our staining results (not shown).

Correlations between the BM MVD and Other Parameters in SM

In the group with SM, a significant correlation was found between the MVD and the infiltration grade (percentage of tryptase⁺ BM MC infiltrates) ($r = 0.74$, $P < 0.05$) (Figure 4). A rough correlation between the MVD and serum tryptase was also found but did not reach statistical significance ($r = 0.42$, $P = 0.06$). The BM MVD did not correlate with the percentage of MCs in BM smears, absolute neutrophil counts, percentage of blood basophils or eosinophils, whole blood tryptase, whole blood histamine, serum histamine, lactate dehydrogenase, fibrinogen, or alkaline phosphatase ($P > 0.05$). There was also no correlation between BM MVD levels and specific clinical findings including skin involvement (UP-like skin lesions), flush, vascular instability, or gastrointestinal ulcerative disease in our SM patients ($P > 0.05$).

Discussion

Recent studies have shown that hemopoietic malignancies exhibit increased BM angiogenesis.^{20–22} SM is a clonal hematological disease that almost invariably af-

Figure 2. MVD in MC infiltrates compared to nonaffected marrow. The figure shows the MVD values for bone-marrow mast-cell infiltrates in comparison to the nonaffected adjacent marrow in patients with SM. As visible, the MVD was higher in the MC infiltrates compared to the nonaffected adjacent BM in all cases ($P < 0.05$).

fects the BM.^{1–4,11–14,24} In the present study, we show that the BM MVD is significantly higher in SM compared to CM/UP or controls. This observation is novel and is in line with the notion that SM is a neoplastic hematological disease.

The increased vessel growth in SM seems to be associated with focal accumulations of neoplastic MCs. In fact, there was a significant correlation between the MVD and grade of MC infiltration (dense MC infiltrates) determined by tryptase immunohistochemistry. Moreover, the MVD was specifically increased in dense focal MC infiltrates, whereas the unaffected adjacent marrow disclosed normal MVD or only a slight elevation. Such a slight increase in the MVD in the nonaffected BM was especially seen in cases with an additional diffuse spread of MCs or a hypercellular marrow (slowly progressing, smoldering disease). Thus, in these cases, the increase in MVD may in part be because of a more diffuse spread of pathological cells in the affected BM. All in all, however, the increase in BM MVD in SM seems to be closely related to the increase in MCs in the BM.

An increased angiogenesis in the BM is frequently found in hematological neoplasms. In this regard it is noteworthy that patients with SM can develop an associated hematological non-MC lineage neoplasm. In such cases it may be impossible to define whether the increase in BM MVD is because of SM or because of the

Figure 3. Immunohistochemical detection of tryptase, CD34, and VEGF in MC infiltrates. Immunohistochemistry was performed by an indirect immunoperoxidase staining technique as described in the text. **A:** The reactivity of an anti-tryptase mAb with neoplastic MCs in a BM infiltrate in a patient with SM. Serial-section staining of the same area (**B**) shows a high density of CD34+ microvessels in this MC infiltrate whereas only a few vessels were found in the nonaffected adjacent marrow. **C:** CD34+ microvessels in a MC infiltrate in another patient with SM. Again, the microvessels are primarily found within the MC infiltrate. As visualized in **D**, the spindle-shaped atypical MCs reacted with an antibody against VEGF.

concomitant hematopoietic malignancy. Therefore, we decided to exclude patients with concordant hematological neoplasms in this study.

A number of angiogenic growth factors have been implicated in tumor-associated outgrowth of microves-

sels.¹⁶⁻¹⁸ One of these factors seems to be VEGF. Because MCs are known to express VEGF,²⁶ one may speculate on a role of this cytokine in microvessel outgrowth in MC infiltrates. In the present study, we were indeed able to localize VEGF-immunoreactive material to MCs in BM

Table 4. Expression of VEGF in BM Cells in Normal Marrow and SM Patients

Cell types	Expression of VEGF detected	
	Normal BM	SM
Megakaryocytes	+++	+++
Myeloid progenitors	++	++
Neutrophil granulocytes	+/-	+/-
Eosinophil granulocytes	-	-
Erythroid progenitors	-	-
Mast cells	n.t.	++

n.t., not tested.

MC infiltrates. The possibility that the antibody applied produced a nonspecific reaction could be excluded by preincubation with a blocking peptide, which resulted in a negative stain. Therefore, we indeed believe that MCs in SM are capable of expressing VEGF. However, apart from VEGF, other angiogenic factors may also be produced by MCs. Likewise, tryptase, which is expressed in MCs and was detected in SM infiltrates, can act as an angiogenic factor.²⁷ Also, MCs are known to express fibroblast growth factors²⁸ that can promote angiogenesis. In addition, various angiogenic factors may be produced by BM accessory cells stimulated by MC products.

Patients with SM may show either an indolent or (rarely) an aggressive clinical course. In addition, patients with SM may suffer from mediator-related symptoms. In the present study, most patients analyzed had indolent SM. Therefore, it was impossible to correlate the MVD levels with the categories of SM in this study. It is noteworthy however, that in patients with smoldering SM and the only patient with aggressive SM, the BM MVD was also elevated. It is also of interest that all three patients with smoldering SM showed relatively high MVD levels compared to the indolent SM group.

In further investigations, we also attempted to correlate the MVD levels with other disease-related clinical and laboratory parameters. However, no correlations were

found when the MVD levels were compared with the percentage of MCs in BM smears, blood cell (differential) counts, whole blood tryptase or histamine levels, lactate dehydrogenase, or alkaline phosphatase. There was also no correlation between BM MVD levels and mediator-related clinical findings or symptoms. Thus, BM angiogenesis may not depend on the systemic spread or action of MC-derived mediators.

The notion that SM is associated with increased BM angiogenesis may have clinical implications. In this regard it is noteworthy that pharmacological inhibitors of angiogenesis can inhibit the growth of neoplastic hemopoietic (leukemic) cells. In summary, our data show that SM is associated with increased BM angiogenesis. This observation may have implications for the pathophysiology of SM and the development of new treatment strategies.

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Figure 4. Correlation between MVD and MC infiltrates. The correlation between the MVD and the infiltration grade (tryptase⁺ dense MC infiltrates) of the BM in patients with SM is shown. The correlation was found to be significant in all calculations ($r = 0.74$; $P < 0.05$).

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