Growth Factors, Cytokines, Cell Cycle Molecules

Evaluation of Angiogenesis and Vascular Endothelial Growth Factor Expression in the Bone Marrow of Patients with Aplastic Anemia

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It is generally appreciated that bone marrow function and growth of myelopoietic cells depends on an intact microvasculature. A pivotal regulator of angiogenesis is vascular endothelial growth factor (VEGF). Here, we describe analysis of VEGF expression and microvessel density in the bone marrow of patients with aplastic anemia by immunohistochemistry. Bone marrow was examined at diagnosis and at the time of hematological remission after immunosuppressive therapy using anti-thymocyte globulin, cyclosporin A, and glucocorticoids or allogeneic stem cell transplantation. At diagnosis, both VEGF expression and microvessel density were found to be significantly lower in aplastic anemia compared to normal bone marrow (aplastic anemia, 1.1 ± 0.7 events per field, versus controls, 5.9 ± 3.0 events per field; $P < 0.05$). In response to successful therapy, VEGF and microvessel density in the bone marrow increased substantially. Serum VEGF levels were also found to be significantly lower at diagnosis in aplastic anemia compared to healthy controls (51 ± 35 pg/ml versus controls, 444 ± 220 pg/ml; $P < 0.05$). VEGF in the serum increased substantially after successful immunosuppressive therapy or stem cell transplantation ($P < 0.05$). Taken together, these data show that aplastic anemia is associated with reduced angiogenesis and reduced VEGF expression. (Am J Pathol 2006, 168:123–130; DOI: 10.2353/ajpath.2006.050034)

Aplastic anemia (AA) is a life-threatening disorder characterized by a deficiency of pluripotent hematopoietic progenitor cells with consecutive bone marrow (BM) aplasia and peripheral pancytopenia.^{1–3} The histological hallmark of the disease is the empty marrow that contains normal amounts of fat cells but is more or less depleted of myelopoietic progenitor cells, erythroid cells, and megakaryocytes.^{3–5} In a subset of patients, pancytopenia and depletion of myeloid cells in the BM are severe, denoting severe aplastic anemia (sAA).^{3–5} When untreated, patients with AA have a grave prognosis.^{6,7} Current treatment options include hematopoietic stem cell transplantation or therapy with anti-thymocyte globulin (ATG) combined with cyclosporine A (CSA) and glucocorticoids.^{8–11} In response to these therapies, the majority of all patients with AA can be cured.^{8–11}

So far, little is known about the pathogenesis of AA. Based on laboratory data and beneficial effects of immunosuppressive therapies, an abnormal function of the immune system has been discussed as contributing to the pathogenesis of AA.^{2,3,8–13} Thus, previous data have supported the notion that cytotoxic T cells may play a role in the development of AA.^{2,3,12,13} More recently, it has been suggested that humoral components of the immune system, including antibodies against cellular proteins, are also involved in the pathogenesis of AA.¹⁴

Another pathogenetic concept proposed in the context of AA, is related to the BM microenvironment, which consists of endosteal cells, macrophages, fat cells, fibroblasts, and microvascular endothelial cells.^{15,16} In fact, it has been suggested that an abnormal (decreased) growth and function of such microenvironmental cells in the BM may contribute to the development of BM aplasia in patients with AA.^{15,16}

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Table 1. Patients' Characteristics and Results of Routine Laboratory Investigations

No.	Diagnosis	Sex	Age at diagnosis (years)	WBC ($\times 10^9/L$)	ANC ($\times 10^9/L$)	Hb (g/dl)	Reti ($\times 10^9/L$)	Plt ($\times 10^9/L$)
01	nsAA	F	48	2.0	1.0	8.0	31	42
02	nsAA	F	58	2.7	1.0	9.5	49	7
03	nsAA	M	34	3.6	2.3	6.2	36	42
04	nsAA	F	37	3.6	2.0	9.6	91	59
05	nsAA	M	25	2.1	0.6	7.0	29	11
06	nsAA	F	66	1.1	na	8.4	32	28
07	nsAA	F	37	3.0	0.8	9.1	82	16
08	nsAA	M	31	2.5	0.7	12.7	53	52
09	nsAA	F	46	3.4	1.7	8.8	22	24
10	sAA	M	65	3.3	1.7	10.5	18	17
11	sAA	F	34	2.1	1.8	8.4	13	3
12	sAA	M	20	1.7	0.2	8.0	14	33
13	sAA	M	24	1.4	0.4	6.9	35	11
14	sAA	M	58	0.8	0.3	6.7	41	7
15	sAA	M	28	2.0	0.2	8.6	17	44
16	vsAA	F	42	2.3	0.1	9.6	10	66
17	vsAA	F	65	1.0	0.0	11.4	10	17
18	vsAA	F	79	2.0	0.1	9.2	17	24

nsAA, nonsevere aplastic anemia; sAA, severe aplastic anemia; vsAA, very severe aplastic anemia; WBC, white blood cell count; ANC, absolute neutrophil count; Hb, hemoglobin; Reti, reticulocytes; Plt, platelets. na, not available.

The microvasculature is an essential component of the microenvironment in diverse organs and has been described as a critical target in various disorders. Likewise, the BM microvasculature is considered to play an important role in hematopoietic neoplasms.^{17–24} Thus, in most BM neoplasms including myeloid leukemias and myelodysplastic syndromes, an enhanced BM angiogenesis has been described.^{17–20,23,24} To a certain degree, the same may hold true for reactive disease states (systemic inflammation, chronic infection) in which enhanced BM function, ie, growth of progenitor cells in BM cavities, is required for the extra production of granulomonocytic effector cells.

A central regulator of BM angiogenesis is vascular endothelial growth factor (VEGF).^{17–22} Among various VEGF species, VEGF-A appears to be expressed most abundantly in normal and neoplastic myeloid cells.^{17–22} Thus, in most instances, the levels of VEGF-A correlate with the extent of angiogenesis, ie, the BM microvessel density (MVD).^{20–22} Within the hematopoietic system, VEGF is considered to be primarily produced in megakaryocytes and immature myeloid cells.^{24,25} Based on this hypothesis, it was of interest to learn whether BM angiogenesis and VEGF expression change in patients with AA. The specific aims of the present study were to examine BM angiogenesis and VEGF expression in patients with AA and to compare these values with those obtained in normal BM. In addition, we examined the effects of immunosuppressive therapy (with ATG, CSA, and glucocorticoids) or allogeneic stem cell transplantation on angiogenesis and VEGF expression.

Materials and Methods

Patients

Eighteen patients with AA were examined in this study. BM specimens were obtained from the posterior iliac

crest in all cases after informed consent was given. Additional diagnostic investigations included physical examination, ultrasound (spleen), complete blood count, and blood chemistry. The patients' characteristics are shown in Table 1. The diagnosis of AA was established according to published criteria.^{26,27} Diagnosis of sAA required a hypocellular BM (cellularity $<30\%$ according to Tuzuner and Bennett²⁸) and two or three of the following abnormal peripheral blood values: absolute neutrophil count $<0.5 \times 10^9/L$, platelets $<20 \times 10^9/L$, or anemia with reticulocytopenia of $<20 \times 10^9/L$. The diagnosis of very severe aplastic anemia (vsAA) was established when sAA criteria were met and absolute neutrophil counts were $<0.2 \times 10^9/L$. Nonsevere AA (nsAA) was defined as pancytopenia with hypocellular BM and peripheral blood counts not fulfilling criteria of sAA. Based on these criteria, 9 of 18 patients (50%) were classified as nsAA, 6 patients (33%) as sAA, and 3 patients (17%) as vsAA. In all but six patients, the BM was re-examined after immunosuppressive therapy (ATG, CSA, and glucocorticoids) or allogeneic stem cell transplantation.

Control BM specimens were obtained from three patients with non-Hodgkin's lymphoma, one with suspected myeloproliferative disease, one with suspected multiple myeloma, one with iron deficiency, and one with a local infection without myeloid hyperplasia. None of the control patients had signs of systemic inflammation or lymphoma-associated B symptoms. All patients gave informed consent before BM puncture.

Immunosuppressive Therapy and Stem Cell Transplantation

Patients were treated with ATG, CSA, and glucocorticoids according to published protocols^{8,9} after written informed consent was obtained. Fourteen of the eighteen patients received horse ATG (15 mg/kg per day i.v. for 5

to 8 days) and one rabbit ATG (10 mg/kg per day for 5 days) as well as CSA per os (to reach a target serum CSA concentration of 100 to 200 ng/ml) and glucocorticoids (to prevent serum sickness). Nine patients received granulocyte-colony stimulating factor (G-CSF) subcutaneously at a dose of 5 μ g/kg per day until neutrophil recovery. In three patients, hematopoietic stem cell transplantation was performed using stem cells from an HLA-matched sibling donor after written informed consent was given.

Histology and Immunohistochemistry

In all patients, the BM was examined by routine histology and cytology at diagnosis. In 12 patients, BM was analyzed before and after successful therapy. Immunohistochemistry was performed on paraffin-embedded, formalin-fixed BM biopsy sections (15 patients) using the indirect immunoperoxidase staining technique as described.^{21,23,24} Endogenous peroxidase was blocked by methanol/H₂O₂. The CD34 monoclonal antibody (mAb) QBEND 10 (diluted 1:100) was purchased from Immunotech (Marseilles, France), an antibody against FVIII-related antigen (FVIII-rAG) from Dakopatts (Glostrup, Denmark) (diluted 1:400), and a rabbit anti-VEGF antibody (diluted 1:50) as well as a VEGF-blocking peptide from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies were diluted in 0.05 mol/L Tris-buffered saline (pH 7.5) plus 1% bovine serum albumin and applied for 60 minutes. In case of VEGF staining, BM sections were pretreated by microwave oven. In selected cases, the anti-VEGF antibody was preincubated with a VEGF-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-carbazole was used as chromogen. Slides were counterstained in Mayer's hemalaun. Before quantification, VEGF-stained BM slides were carefully examined for internal positive controls (myeloid progenitors, megakaryocytes, plasma cells) and negative controls (erythrocytes, mature granulocytic cells). Only those slides that showed a homogenous staining pattern for VEGF and the appropriate result for internal (positive and negative) controls were subjected to quantitative analyses. Apart from hematopoietic cells, vessel-lining cells and stromal cells were also labeled by the anti-VEGF antibody, although most of the VEGF in the normal BM was found to be expressed by myeloid cells. Staining results obtained with the anti-VEGF antibody were quantified using an arbitrary score: 1, no reactivity found; 2, only a minority of nucleated cells reactive; 3, majority of all nucleated cells reacting with anti-VEGF; 4, strong expression of VEGF in most cells. The cellularity of the BM was quantified according to published guidelines.²⁸

Determination of MVD

The BM MVD was determined in CD34-stained BM sections and FVIII-rAG-stained BM sections essentially as

described by Perez-Atayde and colleagues.²² A total of 10 microscopic fields were examined for the number of events (immunoreactive elements identified as cells). The number of events per field and thus the MVD were determined by two independent observers in each case.

Measurement of Serum VEGF Levels

The levels of VEGF in the serum of our patients with AA were quantified by enzyme-linked immunosorbent assay (ELISA). The levels of immunoreactive VEGF-A were determined in 10 patients with AA and in 20 age-matched healthy controls. In six patients with AA, serum samples obtained at the time of hematological remission after successful immunosuppressive therapy were available for VEGF measurements. Serum VEGF-A concentrations were quantified using a commercial ELISA (R&D Systems, Minneapolis, MN). The detection limit of the VEGF-A ELISA amounted to 9 pg/ml. In three patients with AA and three healthy controls, both serum and plasma samples were examined for the content of VEGF-A. In 9 patients with AA and in 11 healthy controls, serum levels of VEGF-D were quantified by a commercial ELISA (R&D Systems). The detection limit of the VEGF-D ELISA amounted to 11 pg/ml.

Statistical Evaluation

The level of significance was determined by standard statistical tests including Kruskal-Wallis (comparison between AA and controls) and the paired Student's *t*-test (comparing results before and after therapy). Results were considered to be significantly different when the *P* value was <0.05. To determine correlations between serum VEGF levels, VEGF expression in the BM, and the BM MVD, linear correlations were applied. In case of multiple comparisons, *P* values were corrected according to the method of Bonferroni.

Results

Evaluation of Angiogenesis in Patients with AA

Confirming previous studies, patients with AA exhibited reduced BM cellularity when compared to normal BM (AA, 7 $\pm$ 6% versus normal BM, 49 $\pm$ 20%; see also Table 2). As assessed by CD34-immunohistochemistry and FVIII-rAG-staining, the BM MVD (events per field) was significantly lower in patients with AA compared to controls (CD34: controls, 5.9 $\pm$ 3.0 versus AA, 1.1 $\pm$ 0.7, *P* < 0.05; FVIII-rAG: controls, 4.7 $\pm$ 1.1 versus AA, 1.1 $\pm$ 0.7, *P* < 0.05) (Table 2, Figure 1). There was no apparent difference in the MVD when patients in various subcategories of AA (nsAA versus sAA/vsAA) were compared (Table 2). Figure 2 shows a BM islet with microvessels in a patient with AA. After successful ATG therapy or allogeneic stem cell transplantation, the MVD increased significantly (*P* < 0.05) (Figure 1).

Table 2. Angiogenesis and VEGF Expression in Patients with Aplastic Anemia

No.	Diagnosis	Bone marrow cellularity*	Events/field (mean) CD34	Events/field (mean) FVIII-rAG	bm VEGF score	Serum VEGF concentrations (pg/ml)
01	nsAA	10	0.5	0.8	2	na
02	nsAA	5	1.1	1.3	1	48
03	nsAA	1	0.9	0.6	1	na
04	nsAA	1	3.0	1.0	1	120
05	nsAA	10	0.7	1.6	2	61
06	nsAA	1	1.4	0.7	1	na
07	nsAA	20	1.6	1.4	2	48
08	nsAA	15	na	na	na	21
09	nsAA	5	na	na	na	19
10	sAA	5	0.8	1.0	1	na
11	sAA	10	0.6	1.2	1	97
12	sAA	5	0.6	2.6	1	17
13	sAA	5	1.9	2.6	1	na
14	sAA	20	1.4	0.7	2	52
15	sAA	5	na	na	na	22
16	vsAA	5	1.5	0.6	1	na
17	vsAA	5	0.6	0.8	2	na
18	vsAA	1	0.3	0.2	1	na

nsAA, nonsevere aplastic anemia; sAA, severe aplastic anemia; vsAA, very severe aplastic anemia; MVD, microvessel density; na, not available.
 *Results are given in percentage of bone marrow section area and refer to a score proposed by Tuzuner and Bennett.²⁸

Detection of VEGF in the Normal BM and in Patients with AA

In normal BM sections, megakaryocytes and immature myeloid (progenitor) cells were found to express VEGF as assessed by immunohistochemistry. By contrast, erythroid (progenitor) cells did not express detectable VEGF (Table 3). The staining reaction was completely abrogated by a VEGF-specific blocking peptide (not shown), thereby confirming the specificity of the staining reaction. In patients with AA, the levels of VEGF in BM sections were significantly lower compared to controls (score reactivity: AA, 1.25 ± 0.45 versus controls, 2.29 ± 0.49 ; $P < 0.05$) (Figure 3). Table 2 shows a summary of VEGF-staining results obtained in patients with AA. In patients with sAA and vsAA, the levels of VEGF in the BM were similar in range compared to patients with nsAA (sAA/vsAA, 1.4, versus nsAA, 1.3). After successful therapy

(ATG/CSA or allogeneic stem cell transplantation), immunoreactive VEGF in BM sections was found to increase significantly ($P < 0.05$) (Figures 3 and 4).

The distribution and intensity of staining of VEGF in the various compartments and lineages of the BM in patients with AA were identical to those found in the normal BM (Table 3). In particular, myeloid progenitor cells, megakaryocytes, and plasma cells were found to express substantial (and comparable) amounts of VEGF in the normal BM as well as in patients with AA, whereas erythroid cells and mature granulocytic cells appeared to be VEGF-negative in all donors examined (Table 3). In case of megakaryocytes, the intensity of staining for VEGF was only compared between normal and nsAA BM

Figure 1. MVD in AA and in controls. The MVD was determined in BM sections of patients with AA ($n = 12$) before and after therapy as well as in normal BM ($n = 4$) by CD34 immunohistochemistry. After successful therapy (ATG plus CSA or allogeneic stem cell transplantation), the MVD increased significantly compared to pretreatment values in AA ($P < 0.05$). Results represent the mean $\pm$ SD of all donors in each group.

Figure 2. Residual angiogenesis in AA. A BM section obtained from a patient with AA was stained with an antibody against CD34. Whereas most of the marrow space showed complete depletion of myelopoiesis with markedly reduced angiogenesis, residual islets of myeloid cells were found to contain microvessels (arrows) that were easily detectable by expression of CD34 as determined by immunohistochemistry.

Table 3. Expression of VEGF in Bone Marrow Cells in Normal Marrow and in Aplastic Anemia

Cell types	Expression of VEGF in various cell compartments in		
	Normal BM	AA before therapy	AA in remission
Megakaryocytes	+++	+++*	+++
Myeloid progenitors	++	++	++
Neutrophil granulocytes	+/-	+/-	+/-
Eosinophil granulocytes	-	-	-
Erythroid progenitors	-	-	-
Lymphocytes	+/-	+/-	+/-

AA, Aplastic anemia; BM, bone marrow.

*Data refer to results obtained in patients with nonsevere (ns)AA.

because in sAA and vsAA almost no megakaryocytes could be detected in the BM at diagnosis.

Serum VEGF Levels in Patients with AA

Serum VEGF-A levels were found to be lower in patients with AA compared to age-matched healthy controls (AA, 51 ± 35 pg/ml versus controls, 444 ± 220 pg/ml; $P < 0.05$) (Figure 5). The levels of measurable VEGF-A were slightly lower in patients with sAA/vsAA compared to those recorded in patients with nsAA (sAA/vsAA, 47 ± 37 pg/ml versus nsAA, 53 ± 37 pg/ml). In three patients with AA and three healthy controls, plasma VEGF levels were determined. Similar to serum VEGF-A levels, the plasma levels of VEGF-A were significantly lower in patients with AA compared to healthy controls (AA, 15 ± 5 pg/ml versus controls, 77 ± 64 pg/ml; $P < 0.05$).

In six patients with AA, we were able to determine serum VEGF-A levels before and after successful therapy with ATG ($n = 5$) or stem cell transplantation

Figure 3. VEGF expression in AA before and after therapy. BM biopsies were obtained in patients with AA ($n = 12$) before and after successful therapy (ATG/CSA or allogeneic stem cell transplantation). In addition, sections of normal BM ($n = 7$) were examined. BM sections were stained with an anti-VEGF antibody. VEGF expression score: 1, no reactivity found; 2, a minority of nucleated cells reactive; 3, majority of all nucleated cells reacting with anti-VEGF; 4, strong expression of VEGF in most cells. Results represent the mean $\pm$ SD of all donors in each group. As visible, immunoreactive VEGF in BM sections was lower in AA (at diagnosis) compared to controls and increased significantly after successful therapy ($P < 0.05$).

Figure 4. Immunohistochemical detection of VEGF in the BM in AA after successful therapy with ATG and cyclosporin A (CSA). BM sections were obtained in a patient with AA before (A) and after (B) successful immunosuppressive therapy with ATG and CSA. Whereas the empty marrow at diagnosis (A) was found to contain very little if any VEGF, the marrow space exhibited considerable amounts of VEGF after therapy (B), which appeared to be due to the increase in cellularity after repopulation with VEGF-containing leukocytes.

($n = 1$). In all six patients examined, VEGF-A levels were substantially higher at the time of hematological remission compared to the time of diagnosis ($P < 0.05$) (Figure 5). To determine whether the decrease in VEGF-A was accompanied by decreased expression of other VEGF species, we measured the serum levels of VEGF-D, a cytokine known to be broadly expressed in mesenchymal and nonmesenchymal cells, in 9 patients with AA and 11 healthy controls. Unlike VEGF-A, however, the levels of VEGF-D did not differ between healthy individuals and patients with AA (AA, 675 ± 211 pg/ml versus controls, 658 ± 301 pg/ml; $P > 0.05$), which was an expected result. Together, these data show that patients with AA exhibit decreased VEGF-A levels compared to healthy controls and that VEGF-A levels increase after successful therapy in these patients.

Figure 5. Serum VEGF-A levels in AA before and after therapy compared to healthy controls. Serum levels of VEGF-A were measured by an ELISA in patients with AA ($n = 6$) before and after successful therapy as well as in healthy controls ($n = 20$). Results represent the mean $\pm$ SD of all donors in each group. As visible VEGF-A levels are significantly lower in AA compared to healthy controls ($P < 0.05$), and increased significantly after successful therapy ($P < 0.05$).

Discussion

A number of previous and more recent studies have pointed to a potential role of VEGF as a mediator of angiogenesis in normal tissues as well as in neoplastic disorders. Likewise, in patients with myeloid neoplasms (leukemias), VEGF levels and BM angiogenesis (MVD) are higher when compared to normal BM.^{17–24} Moreover, it has been reported that VEGF-deficient mice exhibit reduced BM function and reduced angiogenesis.²⁹ However, VEGF deficiency in humans has not been identified so far. We here show that patients with AA exhibit decreased VEGF levels and reduced angiogenesis compared to normal BM. After successful therapy, BM angiogenesis and VEGF levels increased substantially. All in all, these data suggest that AA is associated with reduced angiogenesis and reduced expression of VEGF.

VEGF has recently been implicated in the control of growth and differentiation of most primitive hematopoietic progenitors in mice.²⁹ Notably, VEGF has been described as an (intracellular) autocrine growth regulator of murine hematopoietic stem cells.²⁹ More mature myeloid progenitors also express VEGF and VEGF receptors and thus may use this cytokine as an autocrine factor.³⁰ Our data show that in patients with AA, the levels of VEGF are significantly lower compared to normal BM. However, based on our results, it could not be clarified whether the low VEGF level in AA was a consequence of aplasia (depletion of VEGF-expressing progenitors and megakaryocytes during evolution of AA) or was (in addition) a causative factor triggering BM aplasia. Because the data of Gerber and colleagues^{29,30} obtained with murine stem cells suggest that especially intracellular VEGF is important for the proliferation and repopulation of myeloid progenitor cells, we favor the hypothesis that VEGF depletion is a consequence rather than a disease-triggering factor in patients with AA.

A number of recent studies have shown that VEGF-A is produced in megakaryocytes and myeloid progenitor cells under physiological conditions.^{21,23–25} However,

vascular cells and other microenvironmental cells may also produce and secrete VEGF-A under various circumstances.³¹ Thus, little is known about the relative contribution of leukocyte- or megakaryocyte-derived VEGF to the baseline levels of VEGF-A detectable in the serum of healthy individuals. Our data show that VEGF-A levels are clearly lower in patients with AA compared to age-matched healthy controls. Based on this result, it is tempting to speculate that at least a significant proportion, if not all, of the VEGF-A in the serum of healthy individuals is derived from hematopoietic cells. An alternative explanation would be that certain hematopoietic cells also produce factors, such as cytokines, that regulate the production and/or secretion of VEGF in nonhematopoietic cells. In this regard it is noteworthy that tumor necrosis factor- α and interleukin-1 α , both of which are produced in leukocytes, can induce expression and release of VEGF in mesenchymal cells.^{32,33} Another alternative possibility would be that leukocytes or leukocyte products inhibit the degradation or excretion of VEGF. The possibility that CSA (given to AA patients over many months) or G-CSF (administered to nine of our AA patients) directly induced the expression and secretion of VEGF in hematopoietic or nonhematopoietic cells must also be considered. In the case of CSA, it would be tempting to speculate that the beneficial effects of CSA in the treatment of AA might not be attributable to its immunosuppressive properties alone, but could also derive from its stimulatory effect on VEGF production in hematopoietic or nonhematopoietic cells.^{34,35} However, depending on the cell type and type of disease, CSA may display both VEGF-inducing and VEGF-suppressive activities *in vivo*.^{34–37} With regard to G-CSF, a direct effect of the cytokine on vessel growth must be considered.³⁸ However, the MVD at remission in AA patients receiving G-CSF was within the same range, although slightly lower, compared to patients who did not receive G-CSF. Thus, the MVD increased in all patients with AA independent of the administration of G-CSF. An effect of CSA also seems unlikely but cannot be excluded with certainty because all patients received the drug.

At least four different VEGF species (VEGF-A, -B, -C, and -D) have been described.^{39–42} We were therefore interested in whether the decrease in VEGF-A in AA (compared to normal serum) was accompanied by a decrease in VEGF-D, which promotes lymphangiogenesis and is broadly expressed in diverse neoplastic and nonneoplastic tissues, in contrast to VEGF-A (expressed primarily in myeloid cells, plasma cells, megakaryocytes, and less abundantly in other mesenchymal cells).^{41,42}

However, as expected, the levels of VEGF-D in AA were in the same range as that found in healthy controls. Whether lymphangiogenesis is normal or is reduced in patients with AA remains unknown. Based on our VEGF-D results, it may be concluded that at least the VEGF-D-dependent growth of lymphatic vessels is normal in these patients. Studies are under way to clarify this issue and the potential role of VEGF-D-dependent and -independent lymphangiogenesis in the pathogenesis of AA.

An interesting aspect of this study was that the BM cellularity in our AA patients correlated well with the VEGF

score but did not correlate with MVD in the same way. This discrepancy may have several explanations. The most likely one may be that VEGF is primarily produced by hematopoietic cells in the BM, whereas microvessel growth and thus the MVD may be influenced not only by VEGF but also by other angiogenic cytokines and microenvironmental factors.

AA is a heterogeneous disease in terms of its pathogenesis, severeness, and response to immunosuppressive therapy.^{2–11} In our study, patients with nsAA, sAA, and vsAA were examined. In all three groups of patients, increases in the VEGF levels and in the MVD were seen after successful therapy. An interesting aspect of the study was that the increases were in the same range when comparing nsAA patients with sAA/vsAA patients. Thus, a very severe BM aplasia does not exclude a complete reconstitution of VEGF-expressing cells. On the other hand, it should be noted that only patients who showed a good response to therapy with ATG and CSA or stem cell transplantation were included in the present study.

Patients receiving allogeneic BM transplants may also have developing vessels in their BM because of transplanted CD34⁺ stem cells, as an example of postnatal vasculogenesis. Thus, transplanted stem cells may differentiate into two types of cells, namely specialized white blood cells and maturing endothelial progenitor cells, which work together to create new vessels that support growth of BM progenitors.^{43,44} In these patients, angiogenesis and vasculogenesis may co-exist in the BM and participate together in the increase of vessel counts. On the other hand, conditioning for stem cell transplantation may severely affect the BM microvasculature and thereby may counteract angiogenesis/vasculogenesis. We found that the BM MVD in patients with AA in remission was slightly lower (but not higher) in patients receiving a BM transplant compared to those receiving conventional immunosuppressive therapy (not shown).

In summary, our data show that AA is associated with reduced expression of VEGF in the BM and reduced BM angiogenesis. This observation advances our knowledge about cytokine regulations in AA and may have implications for new concepts relating to the diagnosis and therapy of this disease. However, the exact pathogenetic significance of loss of VEGF in these patients remains to be determined.

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