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Bone marrow angiogenesis and its clinical correlates in myelofibrosis with myeloid metaplasia

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A B S T R A C T

Background and Objectives. Previous retrospective studies have indicated markedly increased bone marrow angiogenesis (BMA) in myelofibrosis with myeloid metaplasia (MMM). This issue is further examined in the current prospective study and clinico-pathological correlates sought.

Design and Methods. This was a prospective single institutional study of 66 patients with bone marrow biopsy-proven MMM who were consecutively accrued. Bone marrow angiogenesis was evaluated by assessing microvessel density through immunohistochemical staining for the CD34 antigen. Laboratory and clinical information was collected concurrently.

Results. The 66 patients (median age 62 years, 46 males) included 36 with fibrotic phase agnogenic myeloid metaplasia (AMM), 6 with cellular phase AMM, 4 with hypocellular variant AMM, 10 with post-polycythemic myeloid metaplasia, and 10 with post-thrombocytopenic myeloid metaplasia. Overall, increased BMA was documented in 61 patients (92%). All of the aforementioned sub-categories of MMM were similarly affected in terms of either grade 3 or 4 BMA but differed in the prevalence of grade 4 BMA (25%, 0%, 0%, 10%, and 10%, respectively). In a univariate analysis the only histological feature that significantly correlated with BMA was the degree of megakaryocyte clustering. Among clinical features, increased BMA was significantly associated with younger age of the patient, lower hemoglobin level, intact spleen, and absence of active therapy. On multivariate analysis, only the latter two retained their significance.

Interpretation and Conclusions. The current prospective study confirms the consistent association of BMA with MMM and suggests that the process starts early, is progressive, and might be dampened by both splenectomy and drug therapy. The study also suggests either a megakaryocyte origin or a megakaryocyte effect for the putative angiogenic cytokine.

Key words: myelofibrosis, myeloid metaplasia, angiogenesis.

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The term myelofibrosis with myeloid metaplasia (MMM) describes a clonal stem cell disorder that is characterized by bone marrow myeloproliferation associated with a polymorphic stromal reaction including myelofibrosis, osteosclerosis, and angiogenesis.¹ The median survival ranges from 2 to more than 15 years depending on the presence or absence of well-defined prognostic factors including age, hemoglobin level, leukocyte count, hypercatabolic symptoms, and circulating myeloblast percentage.^{2,3} Interestingly, neither the degree of bone marrow fibrosis nor spleen size has been significantly correlated with survival.¹ Treatment in MMM is largely palliative and, to date, there is no controlled study that supports a survival advantage from either allogeneic stem cell transplantation or various non-transplant treatment modalities including drug therapy, splenectomy, and involved

field irradiation.¹ MMM can develop *de novo* or arise from antecedent essential thrombocythemia (ET) or polycythemia vera (PV). The usual nomenclature to describe these three disease sub-categories is agnogenic (AMM), post-thrombocytopenic (PTMM), and post-polycythemic (PPMM) myeloid metaplasia, respectively. In addition, a distinct *prefibrotic* or *cellular phase* stage of AMM has been described and accepted in the recent WHO classification.⁴ Most patients in this cellular phase disease are generally asymptomatic with borderline or mild anemia, thrombocytosis, and minimal splenomegaly and tend to survive longer than advanced stage patients.⁴

The contemporary view of MMM pathogenesis envisages a series of events that starts with proliferation of the abnormal stem cell clone, accumulation of clonal megakaryocytes as well as monocytes, intramedullary release of both fibrogenic and

angiogenic cytokines from these clonal cells, and a florid stromal reaction that is manifested as collagen fibrosis and osteosclerosis.^{1,5} In addition, we and others have recently demonstrated, in retrospective cohorts, that bone marrow angiogenesis (BMA) in MMM is substantially increased.⁶ In this study, we prospectively evaluated bone marrow angiogenesis in MMM and sought for clinical and histological correlations with concurrently collected parameters.

Design and Methods

Patients

After obtaining approval from the Mayo Clinic institutional review board, 66 consecutive MMM patients (newly diagnosed as well as previously treated) attending the hematology clinic were enrolled in the study from January 2000 to June 2001. The study cohort met the standard diagnostic criteria for MMM^{1,7} and included all the subtypes: fibrotic phase AMM, cellular phase AMM, PPMM, and PTMM. The baseline work-up included physical examination, laboratory assessment of serum chemistry and hematologic parameters, peripheral blood CD34 count, and bone marrow examination with cytogenetics and fluorescent *in situ* hybridization (FISH) studies to exclude chronic myeloid leukemia. The bone marrow microvessel density (MVD) estimations in patients with MMM were compared with values from bone marrow core biopsy specimens from 5 normal adult patients.

Bone marrow evaluation

Bone marrow biopsy slides were prepared from paraffin-embedded blocks. Marrows were stained with hematoxylin and eosin using standard techniques. In a uniform fashion, each marrow was examined and subsequently graded for cellularity (1-3), presence of reticulin fibrosis (1-4), osteosclerosis (0-3), and presence and degree of megakaryocyte clumping (1-3) based on previously published criteria.⁸ Immunohistochemical staining in paraffin-embedded sections was performed by an immunoperoxidase method with avidin-biotin complex as previously described.⁹ Bone marrow sections from paraffin blocks were cut at 4 microns, deparaffinized in xylene, hydrated in sequential gradients of ethanol, and heat treated with citrate buffer (HIER solution, Bio-Tek, 10 mM, pH 6.0) for 30 minutes. Slides were first incubated with primary antibody to CD-34 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and afterwards with biotinylated secondary antibody (Dako, Carpinteria, CA, USA) followed by incubation with streptavidin/HRPO complex reagent (Sigma, St. Louis, MO, USA). To develop the color reaction, AEC and hydrogen peroxide were added. All staining procedures were performed

Table 1. Clinical and laboratory features of 66 patients with myelofibrosis with myeloid metaplasia at the time of bone marrow study.

Measured Parameters	n.	Median	Range
Age (years)	66	62	32-81
Median time from initial diagnosis (months)	66	19.5	0-221
Hemoglobin (g/dL)	66	9.9	0*-14.9
Leukocyte count (/ μ L)	66	9.3	1.3-109.5
Platelet count ($\times 10^3$ / μ L)	66	217	14-1007
Palpable spleen size (cm) ^o	57	10	0-30
Serum alkaline phosphatase level (U/L)	66	259	87-1066
Peripheral blood (PB) blasts %	66	1	0-43
PB immature ^s myeloid cells %	66	5.5	0-33
PB nucleated red cells %	66	1	0-47
Bone marrow cellularity (%)	65	70	5-100

*'0' denotes transfusion dependency. ^oMaximum distance below the left costal margin. Based on analysis of 57 patients with an intact spleen.

^sDefined as blasts + promyelocytes + myelocytes + metamyelocytes.

by the Ventana ES (320) autostainer (Ventana Systems, Tucson, AZ, USA), at 37-42°C, using buffers and detection reagents supplied by the manufacturer. BMA was graded by both visual grading of MVD and vessel counting as described earlier.⁶ To ensure the accuracy of grading, the BMA staining patterns were evaluated independently by two of the authors in a blinded fashion and average values were taken in case of disagreement.

Statistical analysis

Bone marrow angiogenesis was considered the dependent categorical variable (grades 1-4); correlations with continuous variables were studied by the Kruskal-Wallis method and with other nominal variables using χ^2 statistics including Fisher's exact test. For multivariate analysis of clinical features, the MVD categories were converted into a binary model and tested under a logistic regression analysis. Differences were considered statistically significant at $p < 0.05$. All data were analyzed using Stat view software, version 5.01, (SAS, Cary, NC, USA).

Results

The study cohort consisted of 66 patients (46 males and 20 females) with MMM. The clinical and laboratory characteristics of the patients at the time of the bone marrow study are outlined in Table 1. All subtypes of MMM were represented; fibrotic phase AMM (36 patients), cellular phase AMM (6 patients), hypocellular variant AMM (4 patients), PPMM (10 patients), and

Table 2. Bone marrow histological findings of 66 patients with MMM.

Parameters	n.	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4
Reticulin fibrosis	66	NA*	11	34	15	6
Osteosclerosis	66	28	17	10	11	NA
Microvessel density	66	NA	5	20	30	11
Megakaryocyte cluster density	65	NA	4	18	43	NA
BM cellularity	65	NA	18	3	44	NA

*NA: not applicable implies that the grading system used does not incorporate the specific grade unit.^{8,27}

PTMM (10 patients). Nine patients had previously been splenectomized and 23 patients (35%) were red cell transfusion-dependent at the time of the study. Sixteen patients (24%) were previously untreated. At the time of the study, 11 patients were receiving myelo-suppressive therapy, 11 were receiving non-myelosuppressive therapy, and 44 were not on active treatment.

The bone marrow histological findings at the time of the study are outlined in Table 2. Bone marrow angiogenesis was estimated by both visual count and MVD methods and the results were highly concordant. The visual bone marrow angiogenesis grading (1-4) was subsequently used for all clinical and laboratory correlations (Tables 2-4). In general, bone marrow angiogenesis was significantly greater in MMM patients than in controls (5 patients with no known bone marrow disease; $p < 0.0001$). Sixty-one of the 66 study patients (92%) displayed increased bone marrow angiogenesis that was marked (grade 3-4) in 62%. Among the different sub-categories of MMM, there was a non-significant over-representation of grade 4 bone marrow angiogenesis in fibrotic phase MMM (25% vs 0%, 0%, 10%, and 10%, in cellular phase AMM, hypocellular AMM, PPMM, and PTMM, respectively).

Various clinical and laboratory parameters obtained at the time of bone marrow examination were then studied for their possible correlations with bone marrow angiogenesis. Histological variables studied included bone marrow cellularity, degree of megakaryocyte clustering, megakaryocyte count, degree of megakaryocyte morphologic dysplasia, reticulin fibrosis, and osteosclerosis. Among these, univariate analysis revealed significant direct correlation only with the degree of megakaryocyte clustering ($p = 0.01$; Table 3). A similar univariate analysis identified young age, a

Table 3. Univariate analysis of correlation between bone marrow angiogenesis and histological parameters in 66 patients with myelofibrosis with myeloid metaplasia.

Parameters	n	p value
Reticulin fibrosis	66	0.60
Osteosclerosis	66	0.35
Bone marrow cellularity	65	0.71
Megakaryocyte count	65	0.36
Megakaryocyte cluster density	65	0.01
Megakaryocyte morphology	65	0.68

Table 4. Univariate analysis of correlation between bone marrow angiogenesis and clinical parameters in 66 patients with myelofibrosis with myeloid metaplasia.

Parameters	n	p value
Sex	66	0.42
Age	66	0.04
Disease duration	66	0.16
Subtype of MMM	66	0.25
Hemoglobin	42*	0.05
Red cell transfusion need	66	0.42
Platelet count	66	0.26
Leukocyte count	66	0.33
Peripheral blood (PB) blasts %	66	0.85
PB immature myeloid cells %	66	0.98
PB nucleated red cells %	66	0.50
PB CD34 count	66	0.52
History of splenectomy	66	0.03
Palpable spleen size ^o	57	0.15
Presence of symptoms	66	0.72
Dupriez score	66	0.24
Serum alkaline phosphatase level	66	0.50
Previously treated vs untreated	66	0.07
Presence of myelosuppressive treatment at time of study	66	0.04

*Analysis based on red cell transfusion-independent patients.

^oIn non-splenectomized patients.

lower hemoglobin level, and absence of either active treatment or splenectomy as being significantly associated with increased bone marrow angiogenesis (Table 4). However, only the absence of active therapy and the presence of an intact spleen retained their significance during multivariate analysis. Restricting the aforementioned univariate analyses to fibrotic AMM only ($n = 36$) revealed similar significant associations between increased bone marrow angiogenesis and young age

($p=0.001$), an intact spleen ($p=0.03$), absence of active therapy ($p=0.03$), and the degree of megakaryocyte clustering ($p=0.06$). However, the correlation with hemoglobin level was no longer significant.

Discussion

Angiogenesis plays a critical role in neoplastic development, progression and metastasization and has been shown to be an adverse prognostic factor in many solid tumors.^{10,11} It is becoming increasingly evident that angiogenesis plays a key role in the pathophysiology of hematologic malignancies including acute leukemia, multiple myeloma, and MMM.¹²⁻¹⁴ Many recent reports have implied that angiogenesis in MMM is more pronounced than in other chronic myeloproliferative disorders.¹⁴⁻¹⁶ We have previously demonstrated, in a retrospective setting, that 98% of MMM patients have evidence of increased intramedullary angiogenesis.¹⁴ The current prospective study confirms our prior findings by showing a near-uniform (92%) presence of increased bone marrow angiogenesis in MMM. It also shows that all sub-categories of MMM, including cellular phase and hypocellular variant AMM, are similarly affected by increased bone marrow angiogenesis although perhaps to a lesser degree than fibrotic phase AMM. Many hematologic malignancies with stage-wise evolution, including multiple myeloma and acute myeloid leukemia developing from a myelodysplastic syndrome, have been found to have upregulation of angiogenesis with progression of disease.^{17,18} Consistent with this observation, although all cellular phase AMM patients displayed increased bone marrow angiogenesis, none had grade 4 bone marrow angiogenesis whereas 25% of those with fibrotic phase AMM did so. This is consistent with prior data, which suggest that cellular phase patients have increased sinusoidal density which is less pronounced than that seen in fibrotic AMM.¹⁹ Therefore, it is reasonable to postulate that angiogenesis might be the mother event in the evolution of the stromal reaction in MMM and contributes to disease progression. In other words, bone marrow angiogenesis might represent the earliest histo-morphological event in the temporal progression towards myelofibrosis and osteosclerosis. The observed significant association between bone marrow angiogenesis and the degree of megakaryocyte clus-

tering substantiates our current understanding regarding disease pathogenesis in MMM that promotes the secondary nature of the bone marrow stromal reactions including neo-angiogenesis, fibrosis, and osteosclerosis. The current assumption links the pathogenesis of these processes with the release of a cocktail of functionally pleiotropic cytokines that are derived from the resident clonal megakaryocytes.^{1,5,20}

One of the clinical parameters that correlated, in a univariate analysis, with increased bone marrow angiogenesis was anemia. However, the observed clinical significance was lost during multivariate analysis as well as when the analysis was restricted to fibrotic AMM patients only. On the other hand, both univariate and multivariate analyses identified active drug therapy as well as splenectomy as having an anti-angiogenic effect in the setting of MMM. In regards to the effect from chemotherapy, potential explanations include direct suppression of the clonal cell population that secretes angiogenic cytokines^{21,22} and a direct anti-angiogenic effect that has been described for low doses of chemotherapeutic agents.²³ The negative correlation between splenectomy and bone marrow angiogenesis was more intriguing and could be related to the marked reduction in cytokines derived from extramedullary hematopoiesis with pro-angiogenic properties. In this regard, a correlation between splenomegaly and bone marrow angiogenesis as well as intravascular vascular endothelial growth factor levels has been described in myeloproliferative disorders including MMM.^{6,24,25}

The observations from the current study suggest that myelosuppressive chemotherapy that targets the megakaryocyte-cytokine-endothelial cell axis (angiotherapeutics) in early phase disease may potentially alter disease biology and therefore the course of the disease. A similar benefit might be anticipated from treatment approaches that control splenic extramedullary hematopoiesis. However, additional, preferably longitudinal studies are necessary to validate these observations before contemplating a change in current practice.

All authors: substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; drafting the article or revising it critically for important intellectual content; final approval of the version to be published.

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