Bone marrow stromal cell distribution of basic fibroblast growth factor in chronic myeloid disorders

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Background and Objectives. Basic fibroblast growth factor (bFGF) is a multifunctional cytokine that exerts positive regulation in hematopoiesis and that may also have a role in myelofibrosis and angiogenesis. We used bone marrow immunohistochemical stains to obtain additional insight into the cellular distribution of bFGF in both chronic myeloproliferative diseases (CMPD) and myelodysplastic syndrome (MDS).

Design and Methods. Bone marrow immunohistochemical stains were used to evaluate the cellular distribution of bFGF in 29 patients with CMPD, 31 patients with MDS, and 5 normal controls.

Results. The density of bFGF-expressing stromal cells was markedly increased in 74% of the patients with MDS, compared with in only 3% of those with CMPD. In contrast, the density was markedly decreased in 62% of the patients with CMPD (versus 6% in MDS). The staining pattern in normal controls was similar to that in patients with MDS. The presence or absence of associated bone marrow fibrosis did not influence the particular pattern of bFGF expression in either MDS or CMPD.

Interpretation and Conclusions. These observations suggest that bone marrow stromal cell bFGF expression in patients with CMPD is abnormally decreased and that the particular staining pattern may complement the morphologic distinction between CMPD and MDS.

Key words: bone marrow stromal cells, chronic myeloid disorders, chronic myeloproliferative diseases, myelodysplastic syndrome, hematopathology

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The chronic myeloid disorders are operationally classified into chronic myeloproliferative diseases (CMPD) and the myelodysplastic syndrome (MDS).[1] CMPD are characterized by the overproduction of one or more of the formed elements of blood, whereas the main feature of MDS is ineffective hematopoiesis resulting in peripheral cytopenia. Both CMPD and MDS display clonal myeloproliferation that may be associated with a variety of secondary processes, including collagen fibrosis (myelofibrosis), osteosclerosis, and angiogenesis.[2-4] In general, the bone marrow stromal reaction in these diseases is believed to be a reactive process mediated by cytokines that are released by clonal megakaryocytes or monocytes (or both). The implicated pathogenic cytokines include platelet-derived growth factor, transforming growth factor-beta, and basic fibroblast growth factor.[2]

Basic fibroblast growth factor (bFGF) is a multifunctional cytokine that exerts positive regulation in hematopoiesis[5] and that may also have a role in myelofibrosis and angiogenesis.[6,7] Previous studies have shown that the growth factor is physiologically present in various bone marrow cell types, including megakaryocytes, platelets, granulocytes, and stromal cells.[8] More recent studies have suggested increased expression of bFGF in myeloid progenitor cells and in megakaryocytes of patients with myelofibrosis with myeloid metaplasia.[6,9] In addition, increased plasma levels of bFGF have been reported in patients with essential thrombocythemia, polycythemia vera, or myelofibrosis with myeloid metaplasia.[10,11] In the current study, we used bone marrow immunohistochemical stains to obtain additional insight into the cellular distribution of bFGF in both CMPD and MDS.

Design and Methods

Patients

The study patients included 29 with CMPD, 31 with MDS, and 5 normal controls. The 29 patients with CMPD included 8 with polycythemia vera, 9 with essential thrombocythemia, 7 with agnogenic myeloid metapla-

sia, and 5 with chronic myeloid leukemia. The 31 patients with MDS included 3 with refractory anemia, 2 with refractory anemia with ringed sideroblasts, 3 with refractory anemia with excess blasts, 3 with refractory anemia with excess blasts in transformation, 3 with chronic myelomonocytic leukemia, 8 with the 5q- syndrome, and 9 with MDS with associated bone marrow fibrosis. Clinical and laboratory characteristics of the patients are summarized in Table 1. Confounding effects of specific therapy were avoided by studying only patients who were chemotherapy naïve.

Immunohistochemical staining for bFGF and CD68

Bone marrow biopsy specimens were fixed in B-5 for 2 hours, transferred to neutral buffered formalin, and processed routinely, beginning with decalcification in a formic acid solution for 1.5 hours. Immunohistochemical staining of bFGF in paraffin-embedded bone marrow sections was performed by an immunoperoxidase method using avidin-biotin complex and the rabbit polyclonal anti-human bFGF antibody (Santa Cruz, Delaware, CA, USA). Slides of the bone marrow sections were deparaffinized in xylene, hydrated in sequential gradients of ethanol, and pretreated with heated citrate buffer (10 mM, pH 6.0) for 30 minutes. This was followed by sequential incubation in 1:50 dilution of bFGF antibody for 1 hour, biotinylated secondary antibody for 20 minutes, and avidin-biotin complex reagent (Vecta, Vector Laboratories Inc., Burlingame, CA, USA) for 20 minutes with the use of a TechMate 500 autostainer (Ventana, Tucson, AZ, USA). After induction of a color reaction with 3-amino-9-ethylcarbazole substrate solution (Sigma, St. Louis, MO, USA), the slides were counterstained with hematoxylin.

Immunohistochemical staining for CD68 was performed with a mouse monoclonal anti-human CD68-PGM-1 antibody (DAKO, Carpinteria, CA, USA). After deparaffinization, bone marrow section slides were loaded onto the Ventana ES or Nexes autostainer (Ventana) and treated with protease (Ventana) for 12 minutes prior to immunostaining. The primary antibody was incubated with tissue sections for 32 minutes on 1:200 dilution. Labeled streptavidin-biotin detection chemistry was used for antigen visualization. Sections were counterstained with hematoxylin.

Evaluation of bFGF staining by computerized image analysis

An OPTIMA image analysis system was used to evaluate bFGF expression by computerized image analysis. Three representative areas were selected after an initial visual screening with the light microscope under ×200 magnification. Staining for bFGF was then quantified in

Table 1. Clinical and laboratory characteristics of 29 patients with chronic myeloproliferative disease (CMPD) and 31 with the myelodysplastic syndrome (MDS).*

Disorder	*No. of patients*	*Age (yr)*	*Sex (M/F)*	*Hb (g/dL)*	*WBC (x 10⁹/L)*	*Platelets (x 10⁹/L)*
CMPD						
PV	8	50.5 (27-61)	5/3	14.45 (12.7-17.6)	12.7 (5.3-18.2)	336 (127-630)
ET	9	69 (23-74)	3/6	14.1 (6.9-15.5)	10.2 (8.2-12.3)	966 (565-2360)
CML	5	57 (30-64)	4/1	12.1 (7.3-13.2)	17.3 (9.1-204.3)	352 (214-1091)
AMM	7	57 (38-74)	3/4	11.5 (9.3-14.6)	8.2 (1.4-77.1)	162 (106-402)
MDS						
RA	3	78 (71-91)	3/0	9.7 (8.7-11.1)	3.7 (3.6-5.3)	110 (34-344)
RARS	2	62, 67	0/2	8.1, 9.2	2.4, 3.7	245, 327
RAEB	3	79 (65-87)	2/1	9 (8.8-9.1)	2.9 (1.3-4.1)	71 (39-73)
RAEBT	3	64 (33-80)	1/2	8.3 (8.1-11.0)	2.5 (1.6-5.7)	32 (20-144)
CMML	3	74 (40-78)	3/0	10 (6.7-12.6)	4.8 (1.8-11.1)	87 (67-140)
5q- syndrome	8	71 (54-76)	2/6	9.1 (8.0-11.2)	5.6 (3.3-8.2)	273.5 (65-1642)
MDS-*f*	9	71 (48-81)	6/3	8.8 (7.6-12.0)	4.6 (1-13.2)	66 (27-361)
Normal controls	5	59 (35-73)	4/1	14.5 (14.2-15.8)	7.1 (4.3-8.5)	211 (147-258)

*PV = polycythemia vera; ET = essential thrombocythemia; CML = chronic myelocytic leukemia; AMM = agnogenic myeloid metaplasia; RA = refractory anemia; RARS = RA with ringed sideroblasts; RAEB = RA with excess blasts; RAEBT = RAEB in transformation; CMML = chronic myelomonocytic leukemia; MDS-f = MDS with myelofibrosis; Hb = hemoglobin level; WBC = white blood cell count. *Values denote median and range, and some patients with PV were receiving phlebotomy treatment at the time of laboratory testing.*

each of these three regions, and the mean value was used for comparisons. The area occupied by a strong bFGF stain was determined by computerized pixel counting and expressed as a percentage of the total cellular area after subtraction of fat and trabecular bone. The threshold of bFGF staining intensity was set so as not to include weak megakaryocyte expression.

Statistical analysis

The density of bFGF-expressing stromal cells was graded semiquantitatively as being low (minimal or absent stained stromal cells), intermediate (reticular staining easily appreciated), or high (markedly increased density of stained stromal cells). The proportion of patients in each of these categories among the different chronic myeloid disorders was compared by using the chi squared statistic. In addition, results of the quantitative evaluation by image analysis were compared by using the Mann-Whitney and Kruskal-Wallis tests (StatView software, Version 5.0.1, SAS Institute Inc., Cary, NC, USA).

Results

In bone marrow sections of all five normal controls, stromal cell staining of bFGF was strong and the density of bFGF-expressing stromal cells was either high or intermediate (Table 2). Strong bFGF staining was demonstrated in a reticular pattern, compatible with cytoplasmic staining of stromal cells (Figure 1). In addition, weak bFGF staining was noted in both the cytoplasm of megakaryocytes and the nuclei of vascular endothelial cells (Figure 1). This normal staining pattern for bFGF was also seen in almost all the patients with MDS, regardless of the presence or absence of myelofibrosis (Table 2, Figure 2). In contrast, the bFGF-expressing stromal density was markedly reduced (low) in 62% of the patients with CMPD compared with in only 6% and 0% of the patients with MDS or normal controls, respectively ($p < 0.001$ in comparison with MDS or normal controls) (Table 2). The bFGF staining intensity in megakaryocyte cytoplasm and endothelial cell nucleus was weak in all three groups (Figures 1 and 2). Nuclear staining for bFGF was noted in more than 10% of the megakaryocytes in 10 of the 29 patients with CMPD and in 2 of the 31 patients with MDS.

Table 2. Density of basic fibroblast growth factor (bFGF)-staining bone marrow stromal cells among patients with the myelodysplastic syndrome (MDS), chronic myeloproliferative disorders (CMPD), and normal controls (N).

		MDS		CMPD	
Density	*N*	*without fibrosis*	*with fibrosis*	*without fibrosis*	*with fibrosis*
Low	0	1	1	12	6
Intermediate	2	5	1	9	1
High	3	16	7	1	0
Total	5	22	9	22	7

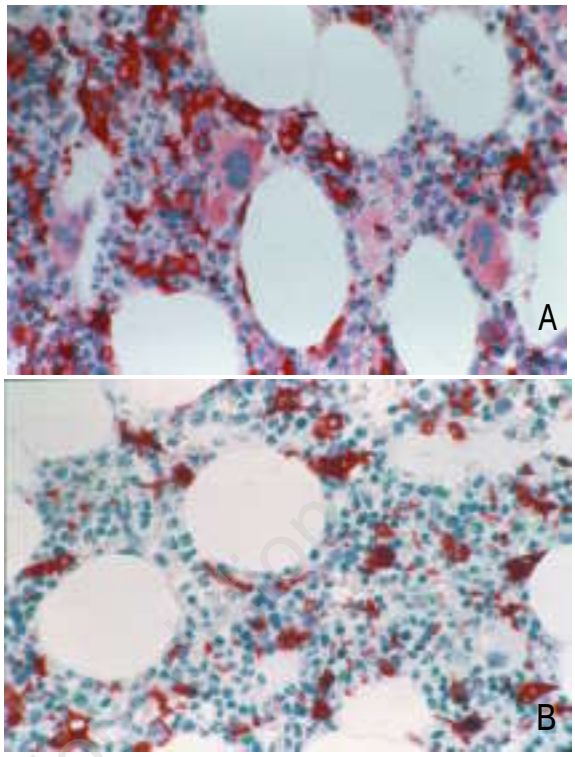

Figure 1. Normal bone marrow immunohistochemical staining for basic fibroblast growth factor (bFGF) and an alternative stromal cell marker (CD68; x128). A, Strong reticular staining pattern for bFGF is compatible with stromal cell expression. B, CD68 stain shows a similar staining pattern. Note weak bFGF expression in megakaryocytes.

The intergroup differences that were determined by the aforementioned semiquantitative method were confirmed by a quantitative computerized image analysis (Figure 3). The percent bFGF staining area, in relation to the overall cellular area of the examination field, in CMPD (mean 0.8, range 0.0-6.0) was significantly lower than that in either normal controls (mean 4.5, range 1.3-7.5, p=0.002) or patients with MDS (mean 4.8, range 0.9-19.1, $p < 0.001$) (Figure 3). The presence of myelofibrosis in both CMPD and MDS was non-significantly associated with decreased percent bFGF staining area (a mean value of 0.5 in agnogenic myeloid metaplasia vs. 0.9 in other CMPD, a mean value of 3.4 in MDS with bone marrow fibrosis vs. 5.5 in MDS without fibrosis). Alternative CD68 staining for histiocytes in representative cases revealed that the altered staining pattern in CMPD was not secondary to a reduced number of histiocytes (Figure 4).

Discussion

The bone marrow bFGF distribution pattern seen in our normal controls is consistent with previous observations that have suggested both the synthesis of bFGF by bone marrow stromal cells and the stromal cell-associated extracellular deposition of the cytokine through heparin-

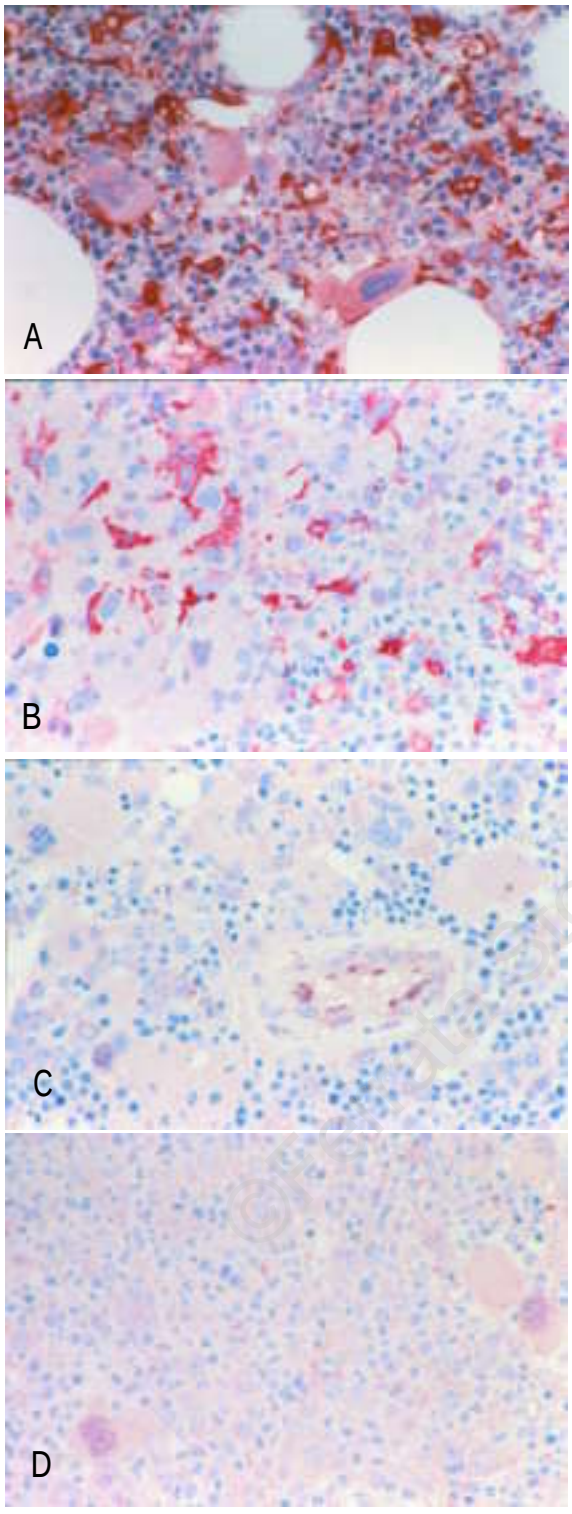

Figure 2. Bone marrow immunohistochemical staining of basic fibroblast growth factor (bFGF) in the myelodysplastic syndrome (MDS) and chronic myeloproliferative disease (CMPD), with or without associated myelofibrosis. (x128.) *A, Refractory anemia. B, MDS with fibrosis. C, Polycythemia vera. D, Myelofibrosis with myeloid metaplasia*. Note decreased stromal bFGF expression in CMPD and weak megakaryocyte bFGF expression in both MDS and CMPD.

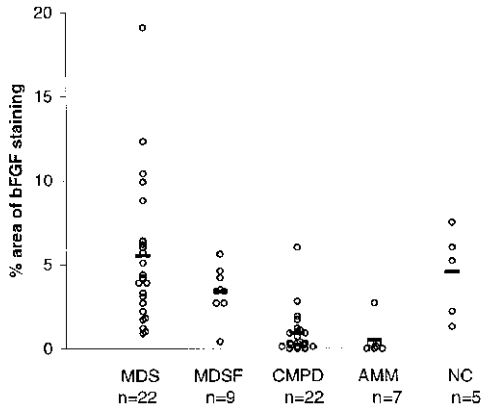

Figure 3. Results of computerized image analysis for basic fibroblast growth factor (bFGF) expression. The marked decrease of bFGF expression in chronic myeloproliferative disorder is again demonstrated. The staining area was non-significantly decreased in the presence of associated myelofibrosis. MDS, myelodysplastic syndrome; MDSF, MDS with myelofibrosis; CMPD, chronic myeloproliferative disorder; AMM, agnogenic myeloid metaplasia; NC, normal controls.

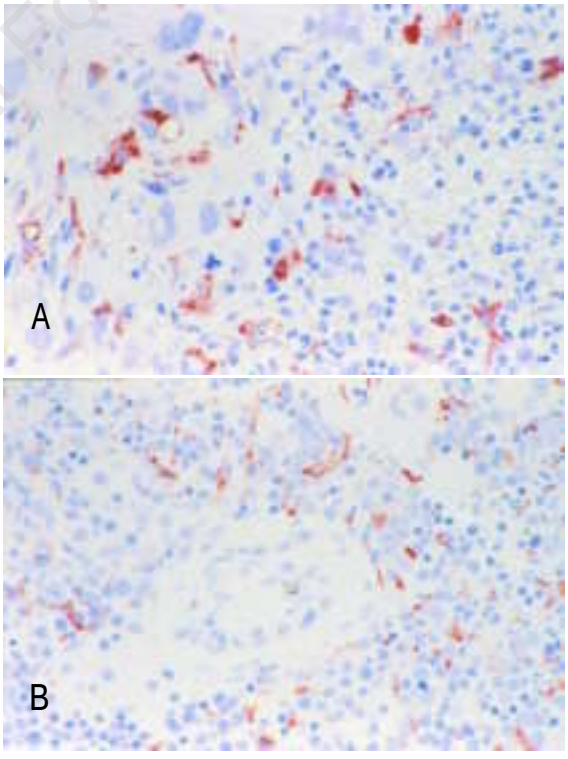

Figure 4. Alternative stromal cell staining with CD68 of areas corresponding to those of Figure 2 B and C. (x128) *A, myelodysplastic syndrome (MDS) with fibrosis. B, polycythemia vera.* These studies showed that stromal cell density was not reduced in chronic myeloproliferative diseases, unlike the findings in MDS.

like bFGF binding sites expressed on the cell surface.[8,12] The current study suggests that this normal mechanism of bFGF production and storage may be intact in MDS but not in CMPD. Possible explanations for this altered stromal distribution of bFGF in CMPD include lower stromal cell concentration, reduced synthesis or retention (or both) of cytokine by stromal cells, and aberrant extracellular deposition of cytokine. The possibility of decreased bone marrow concentration of histiocytes in CMPD as an explanation for the altered bFGF expression pattern was excluded by the demonstration, using a different cell marker (CD68), of abundant histiocytes. Furthermore, previous studies have shown increased stromal cell density in both MDS and CMPD.[13,14] It is, therefore, unlikely that altered cell distribution is the cause of the observed abnormality in CMPD.

bFGF is a potent mitogen for normal stromal cells.[12,15] Because bFGF may also be produced by bone marrow stromal cells, a possible autocrine function has been suggested.[8] In addition, normal stromal cells have previously been shown to express heparin-like extracellular binding sites for bFGF, and this interaction may be functionally important.[8,16] Thus, another possible explanation for the observed abnormality in CMPD may be a defect in either ligand production or cell surface deposition of the cytokine. We are currently planning to approach this issue through bFGF mRNA analysis.

An abnormal extracellular deposition of bFGF might involve an intrinsic defect in the ligand itself[17] or in the ligand-binding molecules. In a preliminary study, we have demonstrated normal receptor density of bFGF both in patients with CMPD and in those with MDS (unpublished data). Therefore, a potential defect in the extracellular binding of bFGF may involve a different set of bFGF-binding molecules, including heparin-like glycosaminoglycans (GAG).[8] However, a GAG-like component of the bone marrow extracellular matrix, hyaluronan, has been shown to be increased both in the circulating plasma and in splenic tissue of patients with myelofibrosis with myeloid metaplasia.[18,19] In any case, the bone marrow composition of GAG in patients with CMPD has not been adequately studied, and a possibly abnormal bFGF-GAG interaction may also involve unrelated soluble molecules.

It is evident from the above discussion that we lack an adequate explanation for the observed difference in stromal cell expression of bFGF between CMPD and MDS. Nevertheless, this difference in immunohistochemical staining pattern may be utilized to complement the morphologic distinction between CMPD and MDS. This feature is especially relevant because the presence or absence of associated myelofibrosis did not influence the pattern of stromal bFGF expression. Among the chronic myeloid disorders, chronic myeloid leukemia is objectively recognized by the demonstration of the Philadelphia chromosome or its molecular equivalent.[20] Similarly, the presence of an elevated red cell mass is relatively specific to polycythemia vera. Essential thrombocythemia, a diagnosis of exclusion, is characterized by persistent thrombocytosis which cannot be attributed to another chronic myeloid disorder. The problem arises when bone marrow morphologic features do not clearly distinguish between myelofibrosis with myeloid metaplasia and MDS with bone marrow fibrosis, on the one hand, and between essential thrombocythemia and MDS with thrombocytosis, on the other.[21,22] The distinction is important because of both prognostic and treatment implications. Nevertheless, the current study involves a relatively small number of patients, and the results should be interpreted with caution.

Finally, megakaryocyte nuclear staining of bFGF was more prevalent in patients with CMPD than in those with MDS. In contrast, endothelial nuclear staining was similar in the two groups. Nuclear localization of bFGF in endothelial cells,[23,24] cultured fibroblasts,[25] and proliferating neuronal cells[26] has previously been recognized, and a functional role in triggering a mitogenic stimulus has been suggested.[27] Nuclear association of bFGF may be restricted to a high molecular weight isoform that may be induced by either cytokine stimulation[28] or stress.[29] In any case, additional studies are required to validate the higher incidence of nuclear bFGF localization in megakaryocytes of patients with Philadelphia-negative CMPD.

Contributions and Acknowledgments

All three authors of the current communication fully and directly participated in the conception, design, data analysis, article drafting, critical revision, and final approval of the study. In addition, all three authors reviewed all pathology slides while the slides were prepared under the supervision of the first author.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

This manuscript was been peer-reviewed by two external referees and by Professor Mario Cazzola, Executive Editor. The final decision to accept this paper for publication was taken jointly by the Editors. Manuscript received September 14, 2000; accepted November 15, 2000.

Potential implications for clinical practice

The observed distinct patterns of bFGF expression revealed by immunohistochemical staining of bone marrow biopsy specimens may: complement histologic distinction between myelofibrosis with myeloid metaplasia and myelodysplastic syndrome with myelofibrosis; complement histologic distinction between essential thrombocythemia and atypical myelodysplastic syndrome with thrombocytosis; suggest the presence of a chronic myeloproliferative disease in a patient with a suspected but not confirmed diagnosis.

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