Overexpression of Basic Fibroblast Growth Factor and Autocrine Stimulation in Acute Myeloid Leukemia

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ABSTRACT

Basic fibroblast growth factor (bFGF) is known to play a critical role in tumorigenesis of solid tumors. The importance of bFGF in hematological malignancies such as acute myeloid leukemia (AML) remains to be elucidated. Therefore, we determined bFGF protein expression by immunohistochemical analyses in bone marrow biopsies of patients with newly diagnosed, untreated AML. The expression of bFGF was significantly increased in AML patients (n = 81; median, 3.0 (interquartile range, 1.8–3.9) arbitrary units (AU)) as compared with controls [n = 18; 1.9 (1.5–2.3) AU]. The degree of bFGF expression did not correlate with microvessel density. bFGF/FGF receptor mRNA and bFGF protein were detected in different AML cell lines. To study autocrine growth stimulation of AML blasts, the AML cell lines HL-60, M-07e, and KG-1 were incubated with bFGF. A significant dose-dependent increase in proliferation and colony formation was observed. These effects were abrogated by the addition of a polyclonal anti-bFGF antibody. In conclusion, increased expression of bFGF in the bone marrow of AML patients seems to play an important role in the pathophysiology of AML by promoting autocrine growth stimulation of leukemic blasts.

INTRODUCTION

bFGF is the prototype member of a family of at least 20 structurally related, heparin-binding growth factors (1, 2). This factor is an important modulator of cell growth and differentiation under both physiological and pathological conditions. The biological activity of bFGF is mediated through a dual receptor system consisting of five high-affinity tyrosine kinase receptors, which display broad expression patterns (3–5), and low-affinity heparan sulfate proteoglycans located at the cell surface and within the extracellular matrix. In this form, bound bFGF constitutes a reservoir of the growth factor that can be released in a regulated manner (6–8). FGF-binding protein mobilizes and activates locally stored FGFs, and this can serve as an angiogenic switch molecule (9, 10). In addition to its extracellular effects, bFGF exerts intracellular effects that seem to be independent from tyrosine kinase-type receptors (2). Moreover, bFGF is mitogenic for endothelial cells and stimulates endothelial cell migration and pericellular proteolysis (11). Furthermore, there is evidence that the mitogenic and angiogenic effects are augmented by VEGF (12–14).

Previous studies suggest a role of bFGF as a prognostic marker for the pathogenesis of hematological malignancies as well (16, 17). High serum bFGF levels were associated with poor survival in patients with non-Hodgkin’s lymphomas (18, 19). In B-cell chronic lymphocytic leukemia, elevated intracellular bFGF levels were found in high-risk patients (20, 21), and the low-affinity bFGF receptor syndecan-1 (CD138) was detected on the surface of myeloma cells (22). Patients with multiple myeloma had significantly higher serum bFGF levels than controls (16), and response to chemotherapy was associated with a significant decrease in bFGF, indicating an important role for bFGF in multiple myeloma as well.

However, little is known about the role of bFGF in AML. bFGF was detected in HL-60 cells and in three of four fresh AML patient samples (23). Furthermore, receptors for FGFs are expressed on some leukemic cell lines such as KG-1 and HL-60 (24, 25). This might suggest that FGF plays a role in leukemic hematopoiesis. Therefore, the goal of our study was to investigate the expression of bFGF in the bone marrow of adult patients with newly diagnosed, untreated AML and to investigate the potential role for bFGF in autocrine growth stimulation of AML blasts.

MATERIALS AND METHODS

Bone Marrow Specimens. Eighty-one consecutive patients (20–79 years old) with newly diagnosed, untreated AML were chosen for our study. This study population is an extension of the previously published cohort of 62 patients in whom the degree of bone marrow angiogenesis at presentation has been reported previously (26). Eighteen unselected controls were taken from the initial group of 22 patients, which have been analyzed for bone marrow MVD as well (26). These controls consist of adult patients (17–77 years old) with various diseases but with normal bone marrow morphology as demonstrated by cytological and histological analyses. From all AML and control patients, a bone marrow core biopsy (iliac crest) for histological diagnosis was obtained at presentation. After every core biopsy, a bone marrow aspiration was obtained through a separate puncture for cytological analyses. All patients had to give written informed consent to the study.

Immunohistochemical Staining. The determination of the degree of angiogenesis was performed by immunohistochemical identification of microvascular endothelial cells with anti-human thrombomodulin antibodies as described previously (27). For bFGF quantification, bone marrow specimens were fixed in paraformaldehyde, decalcified with EDTA, and embedded in paraffin. Serial sections (4–μm thick) of each sample were processed immunohistochemically for the expression of bFGF with rabbit polyclonal antibody (sc-79; Santa Cruz Biotechnology, Santa Cruz, CA; working dilution 1:2000). Controls for immunostaining using nonimmune mouse IgG or rabbit IgG (sc-205 and sc-207, respectively; Santa Cruz Biotechnology) in substitution for the specific first antibodies were consistently negative (data not shown).

Immunohistochemical staining was performed by the alkaline phosphatase/antialkaline phosphatase double bridge technique (Dako-APAAP kit; Dako, Glostrup, Denmark). Briefly, tissue sections were deparaffinized in xylene and rehydrated in a graded ethanol series. Samples were pretreated to promote antigen retrieval in a microwave oven at 450 W twice for 7 min in 10 mM sodium citrate (pH 6.0; Dako). The primary antibodies were applied overnight at 4°C. Subsequent steps were performed according to the manufacturer’s instructions. The fast red substrate (Dako) supplemented with 0.1% (w/v) levamisole was used for revelation of phosphatase activity (30 min at room temperature). Sections were counterstained with 0.1% (w/v) erythrocyan solution.
Evaluation of bFGF Expression. Immunostaining was simultaneously assessed by two independent experienced investigators using light microscopy. The investigators were blinded to the clinical characteristics of the patients and the bone marrow microvessel counts when performing the growth factor evaluation. Expression of bFGF protein was semiquantitatively assessed as described previously (28). Results are reported as AU. In each biopsy sample, expression of bFGF was evaluated in two to three sections processed in independent immunostainings, and the mean value was calculated. To ensure the reliability of the quantification assay, marrow slides have been randomly selected during the study and reanalyzed with excellent agreement. A bone marrow specimen has been used as internal control in the study. Sections of this specimen have been repeatedly immunostained for the same antibodies and quantified by the two investigators at different times.

Blocking studies with the immunogenic peptide that elicited the bFGF antibody (Santa Cruz Biotechnology) demonstrated a substantial reduction of the staining level (>80%) when preincubating the anti-bFGF antibody with the immunogenic peptide in a 100-fold molar mass of the antibody. This observation confirms the specificity of the bFGF antibody.

Cell Culture. The human leukemia-derived cell lines HL-60 (promyelocytic leukemia), M-07e (megakaryocytic leukemia), and KG-1 (myelogenous leukemia) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The HL-60 and KG-1 cell lines were cultured in RPMI 1640 supplemented with 10% FCS (Life Technologies, Inc., Eggenstein, Germany) and 2 mM l-glutamine (Life Technologies, Inc.). The M-07e cell line was cultured in IMDM (Life Technologies, Inc.) supplemented with 20% FCS, 10 ng/ml IL-3 (Sigma, Taufkirchen, Germany), 10 ng/ml granulocyte macrophage colony-stimulating factor (Sigma), and 2 mM l-glutamine at 37°C and 5% CO2. All culture media were supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin.

RNA Extraction, cDNA Synthesis, and RT-PCR. Total RNA from AML cell lines was extracted using RNaseasy total RNA system (Qiagen, Hilden, Germany) following the manufacturer's instructions. cDNA was synthesized for 1 h at 37°C, using 1 μg of total RNA, random hexamers, and Moloney murine leukemia virus reverse transcriptase (Promega, Heidelberg, Germany). bFGF and FGF receptor transcripts were amplified using Taq polymerase (Promega) on a Hybaid thermocycler (MWG-Biotech, Ebersberg, Germany) following a technique described previously (29). Briefly, HL-60 cells were plated at a density of 3 x 10^4 cells/ml in methylcellulose agar consisting of methylcellulose (Sigma, Deisenhofen, Germany), Difco agar (Agar noble; Difco, Detroit, MI), IMDM (Life Technologies, Inc.), 2.5% FCS, and β-mercaptoethanol (Life Technologies, Inc.). This incubation mixture was vortexed thoroughly and kept in the dark at 37°C for 20 min. One ml of this mixture was plated in a tissue culture dish (Nunc). The number of cells finally seeded/dish was roughly 1 x 10^4. To each plate, we added the required concentration of bFGF (final concentration: 0–500 ng/ml) and anti-bFGF antibody (final concentration: 100 ng/ml, 100 μl/dish). The colony numbers were determined by using an inverted microscope after an incubation period of 10 days at pH 7.2, 37°C, in an atmosphere of 5% CO2 and high humidity.

Results. Data are presented as individual data plots or as medians, interquartile ranges (low quartile – high quartile [LQ – HQ]), and ranges. Differences in expression of bFGF between AML and control groups, as well as between AML patients with high and low microvessel densities, were analyzed by the Mann-Whitney rank-sum test for independent groups. Statistical significance of overall differences between more than two groups was analyzed by the Kruskal-Wallis one-way ANOVA. Correlation between variables was assessed by the Spearman rank correlation coefficient (rS). Two-sided P<0.05 were considered significant, and corrections for the number of comparisons (Bonferroni test) were performed when required.

The expression of bFGF was evaluated by immunohistochemical staining in bone marrow core sections of 81 AML patients at first diagnosis. Patient characteristics are depicted in Table 1. The results from these AML patients were compared with the degree of bFGF protein expression in bone marrow biopsies obtained from 18 patients with different diseases but with a normal bone marrow morphology. In the cases of the Hodgkin’s disease, the non-Hodgkin’s lymphomas,
and the kidney carcinoma, the bone marrow was histologically not involved by the underlying disease (Table 1).

The bFGF antigen was consistently detected in every bone marrow specimen from AML, as well as control patients. The staining intensity in the AML bone marrow varied from weak to strong, and the location of the immunoreactivity was extracellular as well as intracellular, as shown in Fig. 1A. Nearly 60% of the AML patients exhibited extensive bFGF staining, whereas 20% were weakly stained for bFGF. In contrast to the AML specimens, the control bone marrow samples displayed a heterogeneous pattern of bFGF staining throughout the cellular areas (Fig. 1B). Both in AML and control specimens megakaryocytes were stained with anti-bFGF antibodies as well. However, these were easily recognized by their characteristic size and morphology. Beside the megakaryocytes, the endothelial cells were weakly or moderately stained for bFGF. The bFGF protein staining scores of the 81 bone marrow specimens from AML patients ranged from 1.0 to 8.5 AU with a median value of 3.0 AU (LQ-HQ: 1.8–3.9), whereas control bone marrow samples (n = 18) showed significantly weaker bFGF protein staining (median, LQ-HQ: 1.9, 1.5–2.3 AU; Mann-Whitney test, P = 0.04; Fig. 2).

The median (LQ-HQ) staining scores for bFGF in relation to the AML FAB subtypes are shown in Table 2. Interestingly, statistical analyses did reveal a significant difference in the expression of bFGF in AML subtypes (Kruskal-Wallis test, P = 0.037). Beside the M3 subtype (three samples only), the M4 subtype showed the highest bFGF values. The staining scores of the M6 subtype were in the range of the controls (Table 2).

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Patients < 60 years showed significant higher bFGF immunoreactivity than patients > 60 years (median, LQ-HQ: 3.4, 2.4–4.9 AU versus 2.3, 1.6–3.5 AU, P = 0.037). However, there was no correlation with gender or the degree of leukemic blast infiltration.

From each bone marrow specimen, adjacent sections to those processed in the present study were immunostained with specific markers of endothelial cells (antihuman thrombomodulin antibodies) for the assessment of MVD. The results for MVD of the subgroup of 62 patients have previously been reported (26). The median MVD in the 81 AML patients was 24 (LQ-HQ: 20.4–28.3) microvessels/×500 field and 12.2 (10.6–14.3) microvessels/×500 field in the 18 control patients, respectively. Thus, the MVD values for the presently larger group of AML patients and the subgroup of the control population were not significantly different from those reported previously (26). The expression of bFGF in the bone marrow was not related to the
MVD levels in the group of AML patients, as well as in the total study population (81 AMLs and 18 control patients). Bone marrows of AML patients with strong angiogenesis (76–100% quartile of the population) showed no different staining scores for bFGF than AML marrows with low MVD (1–25% quartile; marrows with ≥28.8 microvessels/×500 field versus marrows with <19.2 microvessels/×500 field: bFGF, 2.2 versus 2.7 AU, P = 0.401; Mann-Whitney test). Furthermore, we found no correlation between bFGF expression in the bone marrow and the degree of MVD in the entire cohort (r2 = 0.122 for 81 AMLs and 18 control patients, P = 0.285).

bFGF and FGF receptors mRNA was detected by RT-PCR in the different AML cell lines HL-60 (FGF receptor-1, FGF receptor-2, and FGF receptor-4), M-07e (FGF receptor-2, FGF receptor-3, and FGF receptor-4), and KG-1 (FGF receptor-1; Fig. 3). Obviously, the type of FGF receptor expression pattern differed between various cell lines. Furthermore, bFGF protein expression was demonstrated by Western blotting in all three cell lines (Fig. 4). To study bFGF growth stimulation of AML blasts, we investigated the effect of exogenous bFGF on the proliferation of these human leukemia cell lines. bFGF induced an ~2-fold increase in proliferation of all cell lines in a dose-dependent manner (Fig. 5). Furthermore, the stimulatory effect could be inhibited by the simultaneous addition of neutralizing polyclonal anti-bFGF antibodies (Fig. 6).

To further evaluate the role of bFGF on the growth of leukemic blasts, we studied the effect of this cytokine on the colony formation of the HL-60 cell line. Exogenous bFGF dose dependently promoted the degree of colony formation ~2-fold (Fig. 7, Kruskal-Wallis test, P < 0.0001). This stimulatory effect was nearly completely abrogated by polyclonal antibodies against bFGF (Mann-Whitney-Test, P < 0.0001).

Recent studies have shown that increased angiogenesis is a common feature in the bone marrow of AML patients (23, 26, 30, 31). Furthermore, there is growing evidence that the growth factor VEGF and its receptors VEGFR-1 and VEGFR-2 play a key role in tumor neovascularization of hematological malignancies (27, 28, 32–34). Beside VEGF, bFGF is a potent angiogenic inducer that may act synergistically in vitro and in vivo (11, 12, 14, 35, 36). Thus far, only few data about the importance of this growth factor in the pathogenesis of AML have been reported.

The current investigation, based on the immunohistological analyses of bone marrow core biopsies of a large cohort of patients, has clearly documented a significantly increased expression of bFGF in leukemic blasts of patients with newly diagnosed, untreated AML. Until now, there are only a few studies concerning bFGF or FGF receptors in AML, mostly done with cell lines obtaining conflicting results (23, 25, 37–40). One group was able to detect bFGF mRNA in the HL-60 cell line (38), and others found a bFGF message in the HL-60 cell line, as well as in leukemic blasts in 3 of 4 patients (23). However, by the use of a semiquantitative scoring system, our study is the first that demonstrates unequivocally a significant increase of in situ bFGF protein expression in the bone marrow of many unselected patients with AML compared with controls.
Other studies investigated the role of bFGF in AML and myelodysplastic syndrome by measuring bFGF serum or plasma levels or soluble bFGF in supernatants of primary cultured AML blasts (30, 41–43). One group found significantly increased serum bFGF levels in patients with acute and chronic leukemias and myelodysplastic syndrome (30, 43). In contrast to these and our results, however, two of these analyses did not reveal elevated serum or plasma bFGF levels. Thus, the authors argued against a predominant role of this mediator in these malignancies (41, 42). However, these conflicting results are not surprising because elevated levels of FGF are rarely found in biological fluids because of its strong association with cells and the extracellular matrix (44). This pool of FGF constitutes a reservoir of the growth factor that can be released and activated, e.g., by heparanases or by FGF-binding protein in a regulated manner (1, 9, 10). Moreover, by measuring serum levels of bFGF, the high molecular weight forms of this mediator would most likely not be detected because of their strict intracellular location (45). Thus, the advantage of quantifying bFGF by immunohistochemistry is its ability to detect all bFGF isoforms. In contrast, by measuring serum bFGF exclusively, the low molecular weight isoforms of this growth factor that are not cell or matrix bound are detected. This could result in underestimation of the role of bFGF in AML and could explain the previous findings that serum bFGF levels were not elevated in patients with this disease (41, 42).

bFGF is known as a potent activator of endothelial cell proliferation and can thus stimulate angiogenesis. However, the bFGF staining level did not correlate with the microvessel density of the evaluated AML patients, neither for the entire group nor for subgroups (FAB subtype, age, gender, and blast infiltration). An explanation for this unexpected finding may be that bFGF possibly does not act directly in stimulating angiogenesis in AML. First, whereas VEGF receptors are up-regulated and readily detected on endothelial cells of newly formed blood vessels in vivo, this does not appear to be the case for FGF receptors (46). Second, FGFs are stored in the extracellular matrix bound to heparan sulfate proteoglycans and can be mobilized by secretion of heparanases or bFGF-binding protein from tumor cells (9, 10). Because of this complex biology of bFGF, the effect on neovascularization is hardly to predict. Therefore, the lack of a direct correlation between in situ bFGF expression and MVD is not surprising.

The fact that bFGF expression levels were not related to MVD might argue against the hypothesis that bFGF secreted by AML blasts support leukemic cell growth by increasing microvessel density (paracrine mechanism). Therefore, we pursued autocrine growth stimulation mechanisms in cell culture assays. bFGF/FGF receptor mRNA and bFGF protein were expressed in all investigated cell lines (HL-60, M-07e, and KG-1). This is in line with other groups that detected at least one FGF receptor on HL-60 (39, 40, 47). In contrast to these and our results, one group, however, could not find bFGF or FGF receptors on this cell line (25). Moreover, we were able to demonstrate that exogenous bFGF (18-kDa bFGF) stimulates the proliferation and colony formation of various AML cell lines in a dose-dependent manner, thus supporting our findings that functional FGF receptors are present in these cell lines. However, we did not observe a significant difference in the bFGF-induced response among the various cell lines. Thus, the effect of bFGF seems to be independent of the FGF receptor expression pattern in the specific cell line. The specificity of this effect is underlined by the observation that the stimulation of proliferation and colony formation was inhibited by the addition of a polyclonal antibody against bFGF. Thus, the bFGF/FGF receptor pathways seem to play an important role in autocrine growth stimulation of AML blasts.

The observation that the addition of the anti-bFGF antibodies alone (without exogenous bFGF) did not significantly reduce the growth of the leukemic cells seems to argue against a formal autocrine loop. However, the lack of such an inhibitory effect may easily be explained by intracellular bFGF activity not accessible for the neutralizing capacity of the antibodies (intracrine loop; Ref. 45). Furthermore, the source of bFGF in the AML bone marrow may additionally stem from the bFGF pool bound to the extracellular matrix. Thus, AML blasts could release bFGF from this pool by secretion of heparanases or bFGF-binding protein, ultimately leading to their own growth stimulation.

In conclusion, bFGF is overexpressed in the bone marrow of AML patients. The promotion of proliferation and colony formation of AML cell lines by exogenous bFGF points to an important role of autocrine mechanisms by this growth factor in the pathophysiology of AML.
OVEREXPRESSION OF bFGF IN AML

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