Effect of Fibroblast Growth Factor 2 on Stromal Cell-Derived Factor 1 Production by Bone Marrow Stromal Cells and Hematopoiesis

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Background
Reduction of intramedullary hematopoiesis and the development of myelofibrosis and splenic hematopoiesis are frequent complications of clonal myeloid disorders that cause severe morbidity and death and present a therapeutic challenge. However, the pathogenesis of these complications is still unknown. We evaluated the effect of fibroblast growth factor 2 (FGF-2), the level of which is elevated in patients with clonal myeloid disorders, on bone marrow stromal cell expression of stromal cell-derived factor 1 (SDF-1), a chemokine that is essential for normal hematopoiesis.

Methods
Reverse transcription–polymerase chain reaction analysis, immunoblot analysis, and enzyme-linked immunosorbent assays were used to examine effects of human recombinant FGF-2 exposure on SDF-1 expression in mouse stromal MS-5 and S-17 cells. Cocultures of human CD34-positive peripheral blood stem cells or mouse pre–B DW34 cells with mouse stromal cells were used to characterize the functional relevance of the effects of FGF-2 on SDF-1 expression. The in vivo hematologic effects of FGF-2 were determined by systemic administration to mice (n = 10). All statistical tests were two-sided.

Results
FGF-2 reduced constitutive SDF-1 mRNA expression and secretion in stromal cells (SDF-1 levels in supernatants: MS-5 cells cultured for 3 days in medium only versus in medium with FGF-2, 95.4 ng/mL versus 22.2 ng/mL, difference = 73.2 ng/mL, 95% confidence interval [CI] = 60.52 to 85.87 ng/mL; P = .002, two-sided Student’s t test; S-17 cultured in medium only versus in medium with FGF-2, 203.53 ng/mL versus 32.36 ng/mL, difference = 171.17 ng/mL, 95% CI = 161.8 to 180.6 ng/mL; P < .001). These effects of FGF-2 were reversible. FGF-2 compromised stromal cell support of the growth and survival of pre–B DW34 and myeloid lineage cells, and these effects were reversed in part by exogenous recombinant SDF-1α (rSDF-1α) (DW34 pre–B cells recovery on S-17 stromal cells, expressed as a percentage of DW34 cells recovered from medium only: with FGF-2 versus without FGF-2, 27.6% versus 100%, difference = 72.4%, 95% CI = 45.34% to 99.51%, P = .008; with FGF-2 plus rSDF1 versus with FGF-2 only, 60.3% versus 27.6%, difference = 32.7%, 95% CI = 9.35% to 56.08%, P = .034; fold increase in number of myeloid lineage cells after culture on S-17 stromal cells: with FGF-2 versus without FGF-2, 0.25-fold versus 3.8-fold, difference = 3.55-fold, 95% CI = 2.66- to 4.44-fold, P < .001; recovery of myeloid cells on S-17 stromal cells, expressed as a percentage of myeloid cells recovered from medium only: FGF-2 plus rSDF-1α versus FGF-2 only, 76.5% versus 32.4%, difference = 44.1%, 95% CI = 32.58% to 55.68%, P < .001). Administration of FGF-2 to mice reversibly reduced bone marrow levels of SDF-1 and cellularity and induced immature myeloid cell mobilization, extramedullary hematopoiesis, and splenomegaly.

Conclusions
Systemic administration of FGF-2 in mice disrupts normal bone marrow hematopoiesis in part through reduced expression of SDF-1. Thus, endogenous FGF-2 may represent a potential therapeutic target in clonal myeloid disorders characterized by bone marrow failure.

Despite previous assertions to the contrary (1), there is no cause of severe morbidity and death, and a therapeutic challenge immature cells to the peripheral blood, and the development of extramedullary hematopoiesis is a serious complication of clonal myeloid disorders. Patients with these disorders have elevated levels of FGF-2 in their bone marrow. The role of FGF-2 in the pathogenesis of bone marrow failure is unclear.

Study design
In vitro studies in mouse and human cells and an in vivo study in a mouse model.

Contribution
FGF-2 was shown to inhibit bone marrow stromal cell expression of SDF-1, a critical regulator of hematopoiesis, and systemic administration of FGF-2 in mice disrupted normal bone marrow hematopoiesis in part by decreasing SDF-1 expression.

Implications
FGF-2 may represent a potential therapeutic target for bone marrow failure in clonal myeloid disorders.

Limitations
It is not known whether systemic administration of FGF-2 to humans affects SDF-1 expression or whether abnormally high levels of endogenous FGF-2 contribute to the pathogenesis of bone marrow failure in patients with clonal myeloid disorders.

Materials and Methods
Reagents and Cells
Human recombinant FGF-2 was purchased from Peprotech Inc (Rocky Hill, NJ) and R&D Systems (Minneapolis, MN) and was used at a concentration of 50 ng/mL unless otherwise stated. Human recombinant SDF-1α (rSDF-1α) was purchased from R&D Systems and was used at a concentration of 300 ng/mL unless otherwise stated. AMD-3100, a specific antagonist of CXCR4, was obtained from the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program and was used at a concentration of 5 μg/mL. RPMI-1640 medium, heat-inactivated fetal bovine serum (FBS), minimal essential medium alpha modification (αMEM), and heat-inactivated horse serum were purchased from Gibco BRL (Invitrogen Co, Carlsbad, CA). Mouse bone marrow-derived stromal cell lines MS-5 and S-17 (20) were gifts of Drs A. C. Berardi (Ospedale Bambin Gesù, Rome, Italy) and K. Dorshkind (University of California, Los Angeles, Riverside, CA), respectively. Stromal cell lines were maintained in αMEM containing 10% FBS. The mouse pre-B cell line DW34 (9) was obtained from Dr P. Kincade (Oklahoma Medical Research Foundation, Oklahoma City, OK) and was cultured in RPMI-1640 medium containing 10% FBS, 50 μM 2-mercaptoethanol, and 1 ng/mL human interleukin 7 (IL-7) (Peprotech Inc). Human osteosarcoma cell lines MG63 and Saos-2 (obtained from Dr P. Robey, National Institute of Dental and Craniofacial Research, Bethesda, MD) were cultured in Dulbecco’s modified Eagle Medium (Invitrogen Co) containing 10% FBS. Human umbilical vein endothelial cells (HUVECs) were prepared and cultured as previously described (13). Human CD34-positive peripheral blood stem/progenitor cells (PBSCs; >80% CD34-positive cells by flow cytometry) collected by apheresis from healthy donors and frozen at −130 °C until use were obtained from the Clinical Center Blood Bank (National Institutes of Health, Bethesda, MD).

Detection of Stromal-Derived Factor 1 by Enzyme-Linked Immunosorbent Assay and Western Blot Analysis
The enzyme-linked immunosorbent assay (ELISA) for the detection of human and murine SDF-1 in culture supernatants and plasma was performed as previously described (13), using a mouse monoclonal SDF-1 antibody (clone 79018; R&D Systems) for coating the plates and the affinity-purified biotin-labeled goat SDF-1 antibody (R&D Systems) for capture of the antigen. The lower detection limit for SDF-1 was 33 pg/mL. Western blot analysis of cell or tissue extracts for murine SDF-1 was performed as previously described (13), using a rabbit polyclonal antibody against SDF-1 (1 μg/mL, Peprotech Inc) and an affinity-purified, horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin G
RNA Preparation and Reverse Transcription–Polymerase Chain Reaction Analysis

Total RNA was extracted from cells with the use of TRIzol reagent (Molecular Research Center, Cincinnati, OH). Complementary DNA (cDNA) was synthesized from total RNA with the use of a SuperScript Preamplification System (Invitrogen Co) and used for semiquantitative reverse transcription–polymerase chain reaction (RT–PCR) as previously described (13); amounts of cDNA for each RT–PCRs were normalized on the basis of similar amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (13). Primer sequences and the predicted sizes of the amplified products were as follows: SDF-1: 5′-ATGCCCAGTGGCATCCTTCA-3′ (sense) and 5′-TGCTTTGTTGTTCTTCAGGCC-3′ (antisense), 120 bp (13); IL-7: 5′-GAATTTCTCTTACGTACATTCC-3′ (sense) and 5′-CCTGATGTCCTTTGAG-3′ (antisense), 365 bp; flt3 (FMS-related tyrosine kinase 3) ligand: 5′-ACACCTGACTGTTA-3′ (sense) and 5′-ATCTTTAGCAGGTGGTCA-3′ (antisense), 87 bp; and GAPDH: 5′-GCCACCCGAGGACTGGAAGCTGGGC-3′ (sense) and 5′-CATTAGCCATGAGGTTCCACCAC-3′ (antisense), 446 bp (21).

In Vitro Cell Proliferation Studies

The effects of FGF-2 and AMD-3100 on the proliferation of DW34, MS-5, and S-17 cells were assessed as described previously (21). Briefly, cells (2 × 10⁴ MS-5 or S-17 cells per well or 2 × 10⁵ DW34 cells per well) were incubated for 48 hours in complete growth medium that contained or lacked FGF-2 or AMD-3100. [3H]Thymidine deoxyribose was then added (0.5 μCi per well; New England Nuclear; PerkinElmer Inc., Wellesley, MA), and the cells were incubated for another 16 hours. DNA synthesis was measured as the mean counts per minute of triplicate wells, as previously described (21).

Coculture of DW34 Cells or CD34-Positive Progenitor Cells With MS-5 or S-17 Cells

DW34 cell growth on stromal cells was evaluated as previously described (22) with slight modifications. Briefly, feeder layers comprising MS-5 or S-17 cells that had been grown to 80%–90% confluence in six-well plates were incubated with or without FGF-2 for 72 hours. The medium was removed from each well, and CD34-positive PBSCs were suspended in long-term culture medium (αMEM containing 12.5% horse serum, 12.5% FBS, 1 μM hydrocortisone, and 50 μM 2-mercaptopethanol) and applied (1.0 × 10⁶ cells in 2 mL) to stromal cell monolayers that had been preincubated for 72 hours in medium that contained or lacked FGF-2. The MS-5–CD34 and S-17–CD34 cocultures were supplemented with medium that contained or lacked AMD-3100 or FGF-2 and incubated for 3 weeks with weekly replenishment of 2-mL culture medium alone or with the appropriate additives (AMD-3100 or FGF-2). In a separate experiment, we examined the effect of exogenous rSDF-1α on the recovery of CD34-positive cells that were cocultured for 3 weeks with MS-5 or S-17 cells by using a modification of the conditions described above. MS-5 and S-17 stromal cell feeder layers (established on 0.5% gelatin-coated 12-well plates and grown to 80%–90% confluency) were incubated in medium that contained or lacked FGF-2 for 72 hours. The FGF-2–pretreated stromal cell monolayers were then incubated for 2 hours in fresh medium that contained or lacked rSDF-1α (500 ng/mL). PBSCs were applied (1.0 × 10⁵ cells in 1-mL long-term culture medium) onto stromal cell monolayers that had been treated with medium only, with FGF-2 only, or with FGF-2 followed by rSDF-1α. The cocultures were supplemented with medium that contained or lacked AMD-3100, FGF-2 (10 ng/mL), or FGF-2 (10 ng/mL) plus rSDF-1α (500 ng/mL) and incubated for 3 weeks; cocultures were replenished twice per week with 0.5 mL of culture medium alone or with the appropriate additives. At the end of the 3-week incubation, nonadherent and loosely adherent cells were counted and analyzed by flow cytometry. The results are expressed as percentage of cell growth, which was calculated by dividing the mean number of cells per well for cells grown in the presence of AMD-3100, FGF-2, or FGF-2 plus rSDF-1α by the mean number of cells per well (six replicate wells) for cells grown in the absence of these additives and multiplying by 100%.

Flow Cytometry

Nonadherent and loosely adherent cells (six replicate wells) from the long-term cocultures were stained with fluorescein-labeled murine monoclonal antibodies to human CD3, CD14, CD19, CD33, CD34, or CD45 or with fluorescein-labeled isotype-matched murine IgG (BD Biosciences, San Jose, CA). Results of flow cytometry were derived from the analysis of 1.0 × 10⁶ viable (i.e., propidium iodide–negative) cells (from two randomly chosen wells) using a FACScan fluorometer (Becton Dickinson, Franklin Lakes, NJ) and analyzed using CELLQuest software (Becton Dickinson). The experiment was repeated three times.

DNA Microarray Analysis

DNA microarray analysis was performed as previously described (23). Briefly, we used TRizol reagent (Molecular Research Center) to extract total RNA from S-17 cells that had been incubated for 72 hours in medium that contained or lacked FGF-2, and generated cDNAs by reverse transcription from 8 μg of total RNA using oligo(dT) with Superscript II (Stratagene, La Jolla, CA). cDNAs from untreated S-17 cells and from FGF-2–treated S-17 cells were

Secondary antibody (Amersham Pharmacia Biotech, Uppsala, Sweden). Equal protein loading was verified by reprobing the membrane with an actin-specific antibody (C-11 antibody; Santa Cruz Biotechnology, Santa Cruz, CA).
labeled with NH-ester Cy3 and Cy5 dyes, respectively (Amersham Pharmacia Biotech), mixed together, and applied to microarray slides that were spotted with 22,272 mouse cDNA clones (array slides were designated Mm-FCRF-CGENext-v8 and were prepared at the National Cancer Institute core facility, Bethesda, MD). After the slides were incubated at 65 °C overnight and washed twice, the fluorescence intensities of the hybridized spots on the microarray slides were read with the use of a GenePix 4000A scanner (Molecular Devices Corporation, Sunnyvale, CA) at 10-nm resolution, and the variable photomultiplier tube voltage was set to yield the maximal signal intensities with less than 1% (wt/vol) probe saturation. Fluorescence images of the slides were analyzed using GenePix 3.0 (Axon) and mAdB software (NCI, Frederick, MD). After the background fluorescence was subtracted, the average signal intensity for each clone hybridized with cDNA from FGF-2–treated cells was divided by the average signal intensity of the corresponding clone hybridized with cDNA from untreated cells. These ratios were normalized on the basis of the distribution of all cDNA clones in the array. Low-quality measurements (resulting from nonuniform or inappropriate hybridization) were excluded from further analysis and treated as missing values. We performed three separate microarray hybridizations. The overall quality of the microarray analysis was evaluated on the basis of the number of high-quality measurements. For each clone, the ratio of the fluorescence intensity of cells treated with FGF-2 to that of untreated cells was calculated and normalized for each experiment. If at least two of the three microarray hybridizations for each clone delivered high-quality results, the ratios were averaged. Results showing expression ratios (FGF-2–treated/untreated controls) below 0.5 or above 2.0 were considered to be of interest.

Animal Studies
All animal experiments were performed according to NIH guidelines for the care and handling of mice. Six-week-old female BALB/c mice (NCI, Frederick, MD) that were maintained in pathogen-limited conditions received a daily intravenous injection of human recombinant FGF-2 (10 mice per group) for 21 days. Five mice from each group were killed by carbon dioxide administration on day 22 after the start of the injections. The remaining mice were given no further injections and were observed for an additional 23 days until day 45 after the start of injections, when they were killed. Peripheral blood was collected from each mouse when it was killed and was used for automated counting of white blood cells (WBCs) and measurement of SDF-1 content by ELISA and/or immunoblotting. Spleens and livers were also removed from each mouse, weighed, and processed for histologic analysis by Histoserv Inc (Germantown, MD). We also harvested both hind limbs from all mice. To obtain bone marrow, we removed both ends of the femurs and washed out the marrow with 150 μL of lysis buffer (1% Nonidet P-40, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate in PBS) containing a protease inhibitor cocktail (CalBiochem, San Diego, CA) and sodium fluoride (20 mM). The resulting bone marrow cell lysates were used for western blot analysis of SDF-1. Animal studies were performed twice.

Statistical Analysis
The statistical significance of differences between groups was evaluated by the Student’s t test in Excel software (version for Macintosh OS X; Microsoft Corp., Redmond, WA). All statistical tests were two-sided. All outcome variables are expressed as mean values with 95% confidence intervals (CIs).

Results
Stromal Cell-Derived Factor 1 Production and Fibroblast Growth Factor 2 Regulation in Stromal Cells
Immunohistochemical studies have documented the presence of SDF-1 protein in bone marrow endothelial cells, osteoblasts, and stromal cells (13,14). We first examined whether endothelial cells, osteosarcoma cells, and stromal cells in culture secrete SDF-1. Using an ELISA to measure SDF-1 protein, we found that after 72 hours of culture, supernatants from the mouse stromal MS-5 and S-17 and human osteosarcoma MG63 cells had detectable levels of SDF-1, whereas supernatants from HUVECs and the human osteosarcoma Saos-2 cells did not (Fig. 1, A). We next examined the effects of FGF-2 on SDF-1 expression in MS-5, S-17, and MG63 cells. Semiquantitative RT–PCR analysis revealed that MS-5 and S-17 cells exposed to FGF-2 expressed substantially lower levels of SDF-1 mRNA than cells that were not exposed to FGF-2 but that SDF-1 mRNA expression in MG63 cells was only minimally affected by FGF-2 (Fig. 1, B). Immunoblot analysis of cell lysates revealed that FGF-2 reduced SDF-1 protein levels in MS-5 and S-17 cells compared with levels in cells not exposed to FGF-2 (Fig. 1, C). An ELISA revealed that FGF-2 treatment also reduced the amount of SDF-1 secreted into the culture medium by MS-5 and S-17 cells (Fig. 1, D). After 72 hours of incubation, SDF-1 levels in culture supernatants of MS-5 cells cultured in medium only versus in medium with FGF-2 were 95.4 ng/mL versus 22.2 ng/mL, difference = 73.2 ng/mL, 95% CI = 65.02 to 85.87 ng/mL, P = .002; SDF-1 levels in culture supernatants of S-17 cells cultured in medium only versus in medium with FGF-2 were 203.53 ng/mL versus 32.36 ng/mL, difference = 171.17 ng/mL, 95% CI = 161.8 to 180.6 ng/mL, P < .001. A dose–response analysis showed that an FGF-2 concentration of 12.5 ng/mL or higher produced maximal inhibition of SDF-1 secretion by MS-5 and S-17 cells (Fig. 1, E). The proliferation of MS-5 or S-17 cells was only minimally altered after 64 hours of incubation with FGF-2 (data not shown).

We next examined the effect of extended exposure of cells to FGF-2 on SDF-1 expression. MS-5 and S-17 cells cultured for 8 weeks, with periodic splitting, in medium containing FGF-2 continued to express substantially lower levels of SDF-1 protein than cells cultured in medium lacking FGF-2 (Fig. 1, F). The viability and growth of MS-5 and S-17 cells were only minimally affected by 8 weeks of culture in medium containing FGF-2 (data not shown). However, extended exposure to FGF-2 induced uniform morphologic changes in semiconfluent cultures of MS-5 and S-17 cells, which acquired a thinner and more elongated morphology that was observed after 72 hours of incubation with FGF-2 and persisted throughout 8 weeks of culture in the presence of FGF-2 (Fig. 1, G).
Effects of Fibroblast Growth Factor 2 Removal on Stromal Cells

We next evaluated whether the reduction in SDF-1 expression and the morphologic changes induced by exposure of MS-5 and S-17 stromal cells to FGF-2 were reversible. MS-5 and S-17 cells were cultured for 72 hours in medium that contained or lacked FGF-2. ELISA analysis of culture supernatants confirmed our previous observation (Fig. 1, D) that cells exposed to FGF-2 secreted approximately 85% less SDF-1 than cells cultured in medium lacking FGF-2 (Fig. 2, A). Aliquots of cells that had been exposed to FGF-2 for 72 hours were then washed and cultured in medium that lacked FGF-2 (FGF-2 removal) or in FGF-2-containing medium (FGF-2 continuation) for 96 hours. ELISA analysis of culture supernatants collected during the 96-hour incubation revealed that removal of FGF-2 induced a progressive increase in SDF-1 secretion (Fig. 2, A). At the end of the 96-hour incubation, SDF-1 levels in supernatants from cells cultured after removal of FGF-2 (MS-5: 59.35 ng/mL, 95% CI = 55.59 to 63.11 ng/mL; S-17: 253.48 ng/mL, 95% CI = 242.06 to 264.9 ng/mL) were similar to those in supernatants from cells that had never been exposed to FGF-2 (MS-5: 62.9 ng/mL, 95% CI = 58.20 to 67.6 ng/mL; S-17: 346.69 ng/mL, 95% CI = 329.27 to 364.11 ng/mL) but were statistically significantly higher than those in supernatants from cells that were maintained in FGF-2 (MS-5: 6.21 ng/mL, 95% CI = 5.85 to 6.57 ng/mL; S-17: 73.07 ng/mL, 95% CI = 57.81 to 88.33 ng/mL; P < .001). Immunoblot analysis of cell lysates confirmed that MS-5 and S-17 cells that had been previously exposed to FGF-2 for 72 hours and then cultured for 96 hours in medium that lacked FGF-2 expressed SDF-1, whereas those maintained for
the additional 96 hours in medium that contained FGF-2 did not (Fig. 2, B, left panels).

We also examined the effect of FGF-2 removal on SDF-1 expression in MS-5 and S-17 cells that were exposed to FGF-2 for 8 weeks. Immunoblot analysis of cell lysates revealed that MS-5 and S-17 cells that were previously exposed to FGF-2 for 8 weeks and then cultured for 96 hours in medium that lacked FGF-2 expressed SDF-1, whereas those maintained for the additional 96 hours in medium that contained FGF-2 did not (Fig. 2, B, right panels). Removal of FGF-2 from MS-5 and S-17 cells that had been exposed to FGF-2 for 72 hours (left panels) or 8 weeks (right panel) also restored the original cell morphology after 96 hours (Fig. 2, C). These results indicate that FGF-2 inhibits SDF-1 expression and affects cell morphology in stromal cells and that these changes are reversible after the removal of FGF-2.

**Effects of Fibroblast Growth Factor 2 on Stromal Cell Support of Pre-B Cell Proliferation**

Stromal cells can support the proliferation of immature B cells (i.e., pre-B cells) in part because they secrete SDF-1 (9, 20). We therefore examined the effect of exposure of stromal cells to FGF-2 on their ability to support the proliferation of the murine pre-B DW34 cells, which are dependent on either IL-7 or SDF-1 for growth and survival and can be propagated by coculture on certain stromal cell lines in the absence of exogenous growth factors (9). When DW34 cells were cultured for 96 hours in medium alone, more than 90% of the cells died (data not shown). By contrast, DW34 cells that were cocultured on MS-5 or S-17 cell monolayers in medium lacking exogenous IL-7 and SDF-1 proliferated vigorously (i.e., the number of DW34 cells recovered after 96 hours of coculture was 8- to 10-fold higher than the input number of cells) (Fig. 3, A, control). By contrast, statistically significantly fewer DW34 cells were recovered from cocultures grown on MS-5 or S-17 cell monolayers that had been treated with FGF-2 both before and during coculture than from cocultures grown on MS-5 or S-17 cell monolayers that had not been treated with FGF-2 (number of DW34 cells recovered from MS-5 cocultures, expressed as a percentage of DW34 cells recovered from medium only: with FGF-2 versus without FGF-2, 32.3% versus 100%, difference = 67.7%, 95% CI = 36.92% to 98.49%, P = .010; number of DW34 cells recovered from S-17 cocultures, expressed as a percentage of DW34 cells recovered from medium only: with FGF-2 versus without FGF-2, 27.6% versus 100%, difference = 72.4%, 95% CI = 45.34% to 99.51%; P = .008) (Fig. 3, A). Addition of exogenous rSDF-1α to FGF-2-supplemented cocultures statistically significantly enhanced DW34 cell growth compared with cocultures containing FGF-2 alone (MS-5 cocultures: FGF-2 plus rSDF-1α versus FGF-2 only, 32.3% versus 27.6%, difference = 4.7%, 95% CI = 13.4% to 49.90%, P = .36; S-17 cocultures: FGF-2 plus rSDF-1α versus FGF-2 only, 60.3% versus 27.6%, difference = 32.7%, 95% CI = 9.35% to 56.08%, P = .034) (Fig. 3, A). Supernatants from cocultures of FGF-2–treated stromal cells plus DW34 cells contained little or no SDF-1, whereas supernatants from control cocultures contained high amounts of SDF-1 (Fig. 3, B).

Addition of AMD-3100, a specific inhibitor of the SDF-1 receptor CXCR4, during coculture reduced the growth of DW34 cells, and the magnitude of this effect by AMD-3100 was comparable to that of FGF-2 (MS-5 cocultures: AMD-3100 versus FGF-2, 30.35% versus 32.30%, difference = 1.95%, 95% CI = −14.51% to 18.4%, P = .761; S-17 cocultures: AMD-3100 versus FGF-2, 26.70% versus 32.30%, difference = 5.60%, 95% CI = −10.18% to 21.39%, P = .62). These results suggest that the presence of exogenous SDF-1 facilitates the interaction of stromal cells with pre-B cells, and that this interaction is partially mediated by CXCR4.

**Fig. 2.** Effect of fibroblast growth factor 2 (FGF-2) removal on stromal cell production of stromal cell-derived factor 1 (SDF-1) and morphology. **A and B** SDF-1 expression after removal of FGF-2. MS-5 and S-17 cells (70%–80% confluent) were incubated with or without FGF-2 (50 ng/mL) for 72 hours. FGF-2–treated cells were washed and recultured (six replicate cultures) in medium that contained or lacked FGF-2 (50 ng/mL). Culture supernatants were collected from each well at the indicated time points and tested for SDF-1 by enzyme-linked immunosorbent assay (values in [ ) represent the mean values and 95% confidence intervals (error bars) of three replicate cultures from a single experiment and are representative of results from five experiments). Cell lysates, obtained at the end of the 96-hour culture, were tested for SDF-1 by immunoblotting. MS-5 and S-17 cells that had been exposed to FGF-2 for 8 weeks. Immunoblot analysis of cell lysates revealed that MS-5 and S-17 cells that were previously exposed to FGF-2 for 8 weeks and then cultured for 96 hours in medium that lacked FGF-2 expressed SDF-1, whereas those maintained for the additional 96 hours in medium that contained FGF-2 did not (Fig. 2, B, right panels). Removal of FGF-2 from MS-5 and S-17 cells that had been exposed to FGF-2 for 72 hours (left panels) or 8 weeks (right panel) also restored the original cell morphology after 96 hours (Fig. 2, C). These results indicate that FGF-2 inhibits SDF-1 expression and affects cell morphology in stromal cells and that these changes are reversible after the removal of FGF-2.
FGF-2, 40.7% versus 27.6%, difference = 13.16%, 95% CI = -20.14% to 46.48%; \(P = .371\) (Fig. 3, A). However, unlike FGF-2, AMD-3100 caused only a modest decrease in the SDF-1 levels in coculture supernatants compared with control coculture supernatants (Fig. 3, B). FGF-2 and AMD-3100 did not inhibit the proliferation of MS-5, S-17, or DW34 cells (not shown). Because both SDF-1 and IL-7 promote the growth and survival of DW34 pre-B cells (9), we also examined IL-7 expression in MS-5 and S-17 cells. RT-PCR analysis showed that neither MS-5 cells nor S-17 cells expressed IL-7 mRNA (data not shown), providing evidence that FGF-2 indirectly inhibits the growth of pre-B lymphocytes on stromal cell monolayers by reducing stromal cell production of SDF-1 rather than by inhibiting IL-7 production.

Effects of Fibroblast Growth Factor 2 on Stromal Cell Support of Human Myeloid Cells

SDF-1 has been reported to promote the survival of myeloid progenitor cells (16) and, in conjunction with other factors, to contribute to the proliferation of CD34-positive peripheral blood progenitor cells (15). We therefore evaluated the effect of FGF-2 on the ability of stromal cells to support the survival of human CD34-positive peripheral blood progenitor cells (PBSCs) in culture. We observed that CD34-positive PBSCs overlaid onto MS-5 or S-17 stromal cell monolayers formed clusters of round cells that were unattached or loosely attached to the monolayers, which was consistent with a previous report (20). We compared the number of these nonattached and loosely attached cells collected after 3 weeks of coculture with the number of cells used to start the coculture. For cocultures that were maintained in medium alone (control), the number of nonadherent or loosely adherent cells had increased above the input cell number 3.8-fold (95% CI = 3.6- to 4.0-fold) for S-17 cell cocultures and 5.2-fold (95% CI = 4.8- to 5.6-fold) for MS-5 cell cocultures. By contrast, the fold increase in the number of nonadherent or loosely adherent cells was statistically significantly lower for cocultures that were maintained in medium containing FGF-2 or AMD-3100 (S-17 cocultures: with FGF-2 versus without FGF-2, 0.25-fold versus 3.8-fold, difference = 3.55-fold, 95% CI = 2.66- to 4.44-fold, \(P = .001\); with AMD-3100 versus without AMD-3100, 1.93-fold versus 3.8-fold, difference = 1.87-fold, 95% CI = 1.11- to 2.64-fold, \(P = .001\); MS-5 cocultures: with FGF-2 versus without FGF-2, 0.25-fold versus 5.2-fold, difference = 4.95-fold, 95% CI = 3.49- to 6.41-fold, \(P = .001\); with AMD-3100 versus without AMD-3100, 1.9-fold versus 5.2-fold, difference = 3.3-fold, 95% CI = 2.15- to 4.45-fold, \(P < .001\)) (Fig. 4, A). The inhibitory effect of FGF-2 was greater than that of AMD-3100 at 5 \(\mu\)g/mL, a concentration we had found produced the maximal inhibitory effect (data not shown). The reductions in nonadherent or loosely adherent cells induced by FGF-2 and AMD-3100 treatment were not due to a redistribution of the CD34-positive PBSCs to the adherent cell compartment because we found statistically significant reductions in the number of CD45-positive human cells among adherent cells (data not shown). FGF-2 at the concentration used in these experiments (50 ng/mL) was not toxic for MS-5 and S-17 cells, as judged by cell viability after 3 weeks of incubation (data not shown) and did not accelerate cell death or reduce the expression of CXCR4 by CD34-positive PBSCs cultured without stromal cells for 96 hours (data not shown), a result consistent with previous observations (24) (later time points could not be examined because CD34-positive PBSCs do not survive without stromal cells). SDF-1 levels were statistically significantly lower in 3-week culture supernatants from FGF-2–supplemented cocultures compared with control cultures lacking FGF-2 (MS-5 cocultures: FGF-2 versus control, 3.53 ng/mL versus 98.29 ng/mL, difference = 94.76 ng/mL, 95% CI = 88.91 to 99.6 ng/mL, \(P = .001\); S-17 cocultures: FGF-2 versus control, 49.91 ng/mL versus 142.35 ng/mL, difference = 92.44 ng/mL, 95% CI = 77.36 to 107.51 ng/mL, \(P = .001\)) but were less prominently affected by the addition of AMD-3100 (MS-5 cocultures: control versus AMD-3100, 50 ng/mL, \(P = .017\); AMD-3100 versus FGF-2, \(P = .008\); AMD-3100 versus AMD-3100 plus rSDF-1α, \(P = .004\); two-sided Student’s \(t\) test).

Fig. 3. Effects of fibroblast growth factor 2 (FGF-2) on stromal cell support of pre-B cell proliferation. MS-5 and S-17 cell monolayers were cultured for 72 hours with or without FGF-2, the culture supernatants were removed, and DW34 cells were added in culture medium alone or in culture medium that contained AMD-3100 (5 \(\mu\)g/mL), FGF-2 (50 ng/mL), or FGF-2 (50 ng/mL) plus recombinant SDF-1α (rSDF-1α) (300 ng/mL). After 96 hours of coculture, viable (trypan blue–negative) nonadherent or loosely adherent cells were counted (A), and SDF-1 levels in the supernatants were measured by enzyme-linked immunosorbent assay (B). The percentage of growth of DW34 cells was calculated by dividing the number of nonadherent or loosely adherent cells in cocultures grown in the presence of each additive (AMD-3100, FGF-2, or FGF-2 plus rSDF-1α) by the number of nonadherent or loosely adherent cells in cocultures grown in the absence of the additive and multiplying by 100%. Growth of cells in the absence of additives (control) was considered to be 100%. Control: stromal cells precultured in medium alone, stromal and DW34 cells cocultured in medium alone; AMD-3100: stromal cells precultured in medium alone, stromal and DW34 cells cocultured in medium with AMD-3100; FGF-2: stromal cells precultured in medium containing FGF-2, stromal and DW34 cells cocultured in medium with AMD-3100; AMD-3100: stromal cells precultured in medium alone, stromal and DW34 cells cocultured in medium with AMD-3100; FGF-2 plus rSDF-1α: stromal cells precultured in medium containing FGF-2, stromal and DW34 cells cocultured in medium containing FGF-2 and rSDF-1α. Data reflect the means and 95% confidence intervals of six independent replicates. Asterisk indicates—MS-5 cells: control versus AMD-3100, \(P = .013\); control versus FGF-2, \(P = .010\); FGF-2 versus FGF-2 plus rSDF-1α, \(P = .036\); S-17 cells: control versus AMD-3100, \(P = .017\); control versus FGF-2, \(P = .008\); FGF-2 versus FGF-2 plus rSDF-1α, \(P = .034\); two-sided Student’s \(t\) test.
immunosorbent assay (measured by enzyme-linked culture supernatants was counted (Nonadherent viable (trypan with the appropriate additives (AMD-3100 or FGF-2). Nonadherent viable (trypan blue-negative) cells were counted (A); SDF-1 protein in culture supernatants was measured by enzyme-linked immunosorbent assay (B). Control: stromal cells precultured in medium, stromal and CD34-positive progenitor cells cocultured in medium only; AMD-3100: stromal cells precultured in medium, stromal and CD34-positive progenitor cells cocultured in medium containing AMD-3100; FGF-2: stromal cells precultured in medium containing FGF-2, stromal and CD34-positive progenitor cells cocultured in medium containing FGF-2. Data reflect the means and 95% confidence intervals of six independent replicates. Asterisk indicates — MS-5 cells: control versus AMD-3100, P < .001; control versus FGF-2, P < .001; S-17 cells: control versus AMD-3100, P < .001; control versus FGF-2, P < .001; two-sided Student’s t test. C) SDF-1 content in cells. S-17 cell monolayers were pretreated for 72 hours with or without FGF-2 (50 ng/mL), washed, and then incubated for 2 hours in medium that contained or lacked recombinant SDF-1α (rSDF-1α) (500 ng/mL). Cell lysates were assayed for SDF-1 by western blotting; the membranes were reprobed with an antibody against actin to control for equal protein loading (left panel). Culture supernatants were removed from replicate cultures and human CD34-positive progenitor cells were added onto treated or untreated feeders in medium alone or medium containing FGF-2 (10 ng/mL) or FGF-2 (10 ng/mL) plus human rSDF-1α (500 ng/mL). Cocultures were incubated for 3 weeks with replenishment of culture medium alone or with the appropriate additives (10 ng/mL FGF-2, or 10 ng/mL FGF-2 plus 500 ng/mL rSDF-1α) twice per week. Nonadherent viable cells were counted; the results are expressed as percentage of growth of control (cells cultures without additives) considered to be 100 (right panel). The results reflect the means and 95% confidence intervals of three replicates and are representative of results obtained in three independent experiments. Asterisk indicates — control versus AMD-3100, P < .038; control versus FGF-2, P = .049; FGF-2 versus FGF-2 plus rSDF-1α, P < .001; two-sided Student’s t test. (D) Cell-surface markers. Flow cytometric characterization of nonadherent cells recovered after 3 weeks of coculture of CD34-positive progenitor cells onto stromal cell monolayers in medium alone (control) or in medium supplemented with FGF-2 plus rSDF-1α. Cells were stained for human CD45, which identifies all human hematopoietic cells and distinguishes them from murine cells in coculture, and for human CD33, which identifies granulocytic progenitors, monocytes, granulocytes, and mast cells in bone marrow. Results are representative of data obtained in three independent experiments.

AMD-3100, 98.29 ng/mL versus 74.90 ng/mL, difference = 23.39 ng/mL, 95% CI = 13.73 to 33.05 ng/mL, P = .006; S-17 cocultures: control versus AMD-3100, 142.35 ng/mL versus 120.92 ng/mL, difference = 21.43 ng/mL, 95% CI = 4.69 to 38.17 ng/mL, P = .027) (Fig. 4, B).

We next examined whether exogenous rSDF-1α could reverse the FGF-2–induced inhibition of CD34-positive PBSC proliferation in cocultures. To this end, we pretreated stromal S-17 cells for 72 hours with FGF-2 and then incubated them for 2 hours in fresh medium that contained or lacked rSDF-1α. Immunoblot analysis of cell lysates revealed that S-17 cells that were treated with FGF-2 followed by a 2-hour incubation in medium alone expressed less detectable SDF-1 (Fig. 4, C, left) that we suspect was mostly rSDF-1α that was bound to heparan sulfate proteoglycans on the cell surface (25). When we used S-17 cells preincubated with FGF-2 alone or FGF-2 plus rSDF-1α in cocultures with CD34-positive PBSCs, we found that CD34-positive cells that were cocultured for 5 weeks on FGF-2–pretreated S-17 monolayers in medium that contained FGF-2 and rSDF-1α cells grew to statistically significantly higher numbers than those cocultured on FGF-2–pretreated S-17 monolayers grown in medium that contained FGF-2 only (results are expressed as percentage of cell recovery; 100% is the recovery in medium lacking FGF-2 and rSDF-1α with FGF-2 versus medium only, 32.4% versus 100%, difference = 67.6%, 95% CI = 24.46% to 113.87%, P = .034; FGF-2 plus rSDF-1α versus FGF-2, 76.5% versus 32.4%, difference = 44.1%, 95% CI = 32.58% to 55.68%, P < .001) (Fig. 4, C, right). Similar results were obtained using MS-5 cell monolayers (data not shown). These
results provide evidence that FGF-2 compromises stromal cell–supportive functions for CD34-positive progenitor cells by reducing stromal cell production of SDF-1.

Virtually all the nonadherent or loosely adherent cells recovered from the 3-week control cocultures (S-17 stromal cells and CD34-positive cells) or the cocultures that were treated with FGF-2 plus rSDF-1α were cell-surface positive for the human-specific cell markers CD45 (which is expressed by all leukocytes) and CD33 (which is expressed by granulocytic progenitors, monocytes, granulocytes, and mast cells but not by hematopoietic stem cells), indicating that they were derived from the human CD34-positive PBSCs and had undergone myeloid lineage differentiation (17) (Fig. 4, D). Cells positive for CD34, CD3, CD14, or CD19 were not detected (data not shown). Similar results were obtained for 3-week cocultures of MS-5 stromal cells and CD34-positive PBSCs (data not shown). These observations demonstrate that, in vitro, FGF-2 impairs the ability of MS-5 and S-17 stromal cells to support differentiation and survival of myeloid lineage cells derived from human CD34-positive cells and confirm that SDF-1 and CXCR4 contribute to this supportive function.

Effects of Fibroblast Growth Factor 2 on Gene Expression in Stromal Cells

Our results showing that rSDF-1α incompletely rescued stromal cell–supportive functions inhibited by FGF-2 (displayed in Figs 3, A, and 4, C) suggested that FGF-2 may have other effects on stromal cells in addition to its inhibitory effect on SDF-1 expression. Therefore, we used microarray analysis to screen S-17 cells grown in the presence and absence of FGF-2 for genes whose expression is regulated by FGF-2. Table 1 provides a subset of results arbitrarily selected to include genes previously linked to regulation of hematopoiesis. The entire list of genes whose expression was substantially increased or decreased by FGF-2 is shown in Supplementary Table 1 (available online). FGF-2 minimally altered S-17 cell expression of many genes previously linked to hematopoiesis, such as ß3 ligand, granulocyte colony-stimulating factor (G-CSF), Stem Cell Factor, and IL-3, but substantially reduced expression of SDF-1 (also named Cxcl12; Table 1). In addition, FGF-2 substantially reduced expression of the genes that encode insulin-like growth factor 1, chemokine (C-C motif) ligand 19, and chemokine (C-X-C motif) ligand 14 and substantially enhanced the expression of gremlin 1, which encodes an antagonist for bone morphogenetic proteins that could regulate hematopoiesis (26–29). Also, FGF-2 substantially reduced expression of procollagens I, II, IV, V, and VIII (Table 1). These results confirm that FGF-2 reduces CXCR4 gene expression in stromal cells and indicate that FGF-2 modulates the expression of other genes that have been previously linked to regulation of hematopoiesis.

Table 1. Gene expression profiling of S-17 cells after treatment with fibroblast growth factor 2 (FGF-2)

<table>
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<tr>
<th>Gene name</th>
<th>Gene description</th>
<th>UniGene code</th>
<th>Expression ratio (FGF-2–treated/control)</th>
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Hematologic Effects of Systemic Fibroblast Growth Factor 2 Administration in Mice

We next examined whether the administration of FGF-2 to mice altered hematopoiesis. Groups of 10 mice received daily intravenous injections of FGF-2 (5 µg per mouse) or vehicle (PBS) for 21 days, at which time half the mice in each group were killed. The remaining mice were observed untreated for an additional 24 days, and then they were also killed. We observed no toxic effects during or after FGF-2 treatment. At the end of the 21-day treatment period, the spleens from FGF-2–treated mice were statistically significantly (P = .003) larger than those from the control mice (Fig. 5, A). Histologic examination of the spleens revealed that FGF-2–treated mice had less white pulp and more red pulp than vehicle-treated mice, which suggests increased extramedullary hematopoiesis in the FGF-2–treated mice (Fig. 5, B). The livers of FGF-2–treated mice displayed occasional clusters of hematopoietic cells, which were largely absent from the livers of vehicle–treated mice (Fig. 5, B). Other organs, including the kidneys, heart, lungs, and intestines, from FGF-2–treated mice appeared normal (data not shown). Bone marrow sections from FGF-2–treated mice displayed thickened bone trabeculae (Fig. 5, B). Although cell density within the marrow cavity was reduced in FGF-2–treated mice compared with that in vehicle–treated mice, there was no apparent bone marrow fibrosis detectable by silver staining (Fig. 5, B). Peripheral blood smears from FGF-2–treated mice contained statistically significantly more immature myeloid cells (particularly myelocytes and metamyelocytes) than those from control mice (Fig. 5, C) (19.7 immature myeloid cells/500 leukocytes versus 3.10 immature myeloid cells/500 leukocytes, difference = 16.6 immature myeloid cells/500 leukocytes, 95% CI = 12.55 to 20.67 cells per 500 leukocytes, Pc.001) but statistically significantly fewer WBCs (4279.5 cells/µL versus 1090.8 cells/µL, difference = 3188.7 cells/µL, 95% CI = 1326.9 to 5050.5 cells/µL, P = .034). Western blot analysis revealed that bone marrow from FGF-2–treated mice expressed less SDF-1 than bone marrow from vehicle–treated mice (Fig. 5, D). Circulating SDF-1 levels, as measured by ELISA, were lower in FGF-2–treated mice than in control mice, but the difference was not statistically
Fig. 5. Evaluation of mice treated systemically with fibroblast growth factor 2 (FGF-2). Mice received intravenous daily injections of phosphate-buffered saline (PBS; control) or 5 µg of human recombinant FGF-2 in PBS via the tail vein for 21 days (10 mice per group). On day 22 after the start of the injections, five randomly chosen mice from each group were killed. The remaining mice were maintained for 23 days without receiving any additional injection and were killed on day 45 after the start of the injections. A) Gross morphology of representative spleens removed on day 22 from FGF-2–treated and control mice (left). The results in the bar graph reflect the mean spleen weight and 95% confidence intervals of five mice per group (right). Asterisk indicates—P = .003; two-sided Student’s t test. B) Microscopic histology of hematoxylin-eosin (H & E) or silver-stained spleen, liver, and bone marrow removed on day 22 from control and FGF-2–treated mice. The size of the white pulp (blue) in the spleen from the FGF-2–treated mouse is reduced compared with that in the control spleen; the size of the red pulp (purple) in the spleen from the FGF-2–treated mouse is increased compared with that in the control spleen. The liver of a mouse treated with FGF-2 shows evidence of extramedullary hematopoiesis (arrows). The bone trabeculae (arrows) in bone marrow from a FGF-2–treated mouse are larger than those from a control mouse. The images shown are representative of the results observed for all mice in the treatment group. C) Analysis of peripheral blood obtained on day 14 after the start of injections. Peripheral blood smear (left) from an FGF-2–treated mouse obtained on day 14 after the start of injections showing the presence of immature granulocytes (dark blue doughnut-shaped cells) and immature red cells (light blue) (original magnification x60). Determination of the number of immature granulocytes in the peripheral blood of FGF-2– (five mice) and control-treated mice (five mice) (right). The results are expressed as the mean number of immature granulocytes per 500 leucocytes and 95% confidence intervals. Asterisk indicates—P<.001; two-sided Student’s t test. D) Western blot analysis of stromal cell-derived factor 1 (SDF-1) content in the bone marrow of control and FGF-2–treated mice obtained on day 22 after the start of injections. The results (representative of three of five mice randomly chosen from each group) reflect immunoblotting with anti-SDF-1 antibodies and reprobing with anti-actin antibodies (to control for equal protein loading per lane). E) Gross morphology of representative spleens (left) and mean weights and 95% confidence intervals of spleens (right) from all control and FGF-2–treated mice obtained on day 45 after the start of injections (23 days after the injections were stopped). F) Representative images reflecting the microscopic histology of spleen and bone marrow from control and FGF-2–treated mice on day 45. G) Immunoblot analysis of SDF-1 content in bone marrow obtained on day 45 from three mice from each group. The membranes were probed with an anti-actin antibody to control for equal protein loading.

Discussion

FGF-2 and other structurally related polypeptides that activate FGF receptor tyrosine kinases are potent inducers of growth,
In this study, we identify FGF-2 as an inhibitor of SDF-1 expression in bone marrow stromal cells and provide evidence that FGF-2 can alter normal bone marrow hematopoiesis in part by decreasing the expression of SDF-1. It was previously reported that FGF-2 modulates various functions of hematopoietic progenitor cells in vitro, such as myelopoiesis and megakaryopoiesis (24,30–33). Among studies that used long-term coculture with primary stromal cells, one study (30) reported that FGF-2 enhances myelopoiesis at low concentrations only (i.e., 0.2–2 ng/mL), whereas other studies reported that FGF-2 either inhibits (34) or only minimally affects the number of granulocyte-macrophage colony-forming units (CFU-GM) (32). In semisolid culture systems that use methylcellulose or agar, FGF-2 in combination with other growth factors was found to slightly increase the number of CFU-GM from purified hematopoietic progenitor cells in some studies (24,31) but not in others (33,34). Our results extend previous findings by showing that FGF-2 alters hematopoiesis at least in part by reducing SDF-1 secretion by bone marrow stromal cells.

SDF-1 and its receptor, CXCR4, have emerged as critical regulators of hematopoiesis because they support the growth and survival of hematopoietic and myeloid progenitor cells and pre-B cells and help to retain immature blood cells in the bone marrow (9,10,12,15,16). For example, in one study (12), lethally irradiated recipient mice that were reconstituted with CXCR4-deficient fetal liver cells had reduced numbers of B lymphocytes and myeloid lineage precursors in their bone marrow but abnormally high numbers of immature B lymphocytes and neutrophil lineage cells in peripheral blood. These findings suggested that CXCR4 inactivation promotes hematopoietic cell mobilization (12). Indeed, drugs that functionally inactivate CXCR4 promote the release of hematopoietic progenitor cells from the bone marrow to the peripheral blood (18). In addition, G-CSF, which is the most commonly used agent to mobilize hematopoietic progenitor cells from the bone marrow to the peripheral blood, is believed to produce this effect by disrupting SDF-1/CXCR4-mediated retention of hematopoietic progenitor cells in the bone marrow (17,19). Furthermore, patients with a syndrome characterized by warts, hypogammaglobulinemia, immunodeficiency, and myelokathexis (WHIM) syndrome are neutropenic because they have a reduced capacity to release mature myeloid cells from the bone marrow to the peripheral blood, a defect that has been attributed to genetic mutations of CXCR4 leading to an abnormal increase in CXCR4 function (35).

Here we describe a new phenotype that was induced by systemic administration of FGF-2 to mice for 3 weeks. The phenotype is characterized by the presence of immature myeloid cells in peripheral blood, which was associated with a decreased level of bone marrow SDF-1. In addition, these mice displayed increased extramedullary hematopoiesis in the spleen and liver, splenomegaly, and a decrease in bone marrow cellularity. Remarkably, some patients who have clonal myeloid disorders and abnormally elevated levels of FGF-2 typically display similar manifestations, i.e., the presence of circulating immature myeloid cells, extramedullary hematopoiesis in the liver and spleen, and a decrease in hematopoietic cells in the bone marrow (1,4,5). Also, prolonged treatment with G-CSF, which, like FGF-2, reduces SDF-1 expression in bone marrow, induced sustained mobilization of immature cells from the bone marrow to the peripheral blood and promoted extramedullary hematopoiesis in a neutropenic patient with B-cell lymphoma (36). These observations support the possibility that FGF-2 may play a role in the pathogenesis of splenomegaly with extramedullary hematopoiesis and reduced bone marrow hematopoiesis in patients with clonal myeloid disorders who display abnormally elevated levels of FGF-2.

In addition to having evidence of bone marrow failure and extramedullary hematopoiesis, some patients with clonal myeloid disorders display thickened bone trabeculae (osteosclerosis), increased bone marrow vascularization with dilation of sinusoids and intravascular hematopoiesis, and medullary fibrosis (1,5). We found that administration of FGF-2 to mice resulted in a thickening of the bone trabeculae, which was likely a direct consequence of the well-characterized bone morphogenic function of FGF-2 (8), but did not induce neovascularization or fibrosis in the bone marrow. Although FGF-2 is a proangiogenic factor (8), recent studies suggest that it cannot induce long-lasting vascular networks alone but requires PDGF or vascular endothelial growth factor (VEGF)-A (37), levels of which have been reported to be elevated in patients with clonal myeloid disorders (5). We examined the possibility that increased collagen deposition in some patients with clonal myeloid disorders may also be attributable to abnormally increased levels of FGF-2 by testing whether FGF-2 augments stromal cell expression of procollagen molecules. Previous studies have shown that FGF-2 promoted fibronectin production by hairy-cell leukemia cells (38). However, we have now determined by microarray analysis that FGF-2 decreases stromal cell expression of procollagen types I, II, IV, V, and VIII, suggesting that FGF-2 is not involved in the pathogenesis of collagen deposition seen in patients with clonal myeloid disorders and abnormally increased FGF-2 levels (5,39).

Indeed, other studies have concluded that TGF-β1 likely plays a pathogenetic role in myelofibrosis associated with clonal myeloid disorders (1,5). However, TGF-β1 does not induce stem cell mobilization or splenomegaly (7) but rather has been proposed to enhance the retention of hematopoietic progenitor cells in the bone marrow (40,41).

Bone marrow stromal cells regulate hematopoiesis by complex mechanisms, including through their physical interactions with the hematopoietic cells and their secretion of many factors (14). We found that FGF-2 reduced the supportive functions of stromal cells by affecting more than SDF-1 production, as evidenced by the observation that FGF-2–treated stromal cells failed to support long-term growth and survival of peripheral blood stem cells to a greater degree than AMD-3100–treated stromal cells and by the failure of exogenous rSDF-1α to fully reconstitute FGF-2 inhibition of stromal cell–supportive functions for hematopoietic cells. Our microarray analysis revealed that genes whose expression is commonly associated with the support of hematopoiesis were not substantially regulated by FGF-2 treatment of stromal cells, including flt3 ligand, which was reported to be abnormally elevated in the bone marrow of patients with various forms of bone marrow failure (42). However, our microarray analysis showed that FGF-2 substantially reduced the expression of IGF1, which can stimulate hematopoiesis, lymphopoiesis, and the formation of erythroid...
burst–forming and colony-forming units (29,43), and promoted expression of gremlin 1, an antagonist of bone morphogenic proteins, which can stimulate hematopoiesis (26). We also found that FGF-2 reduced expression of CCL19 and CXCL14, chemokines that promote monocyte chemotraction (27,28), and may thus regulate hematopoietic cell chemotaxis. CCGR7, the CCL19 receptor, is expressed in the B-cell lineage, but the receptor for CXCL14 is currently undefined (28,44).

Our study has a limitation. The results presented here demonstrate that exogenous FGF-2 profoundly and reversibly compromises murine bone marrow stromal cell support for hematopoietic progenitor cells and promotes extramedullary hematopoiesis in mice. We do not know whether FGF-2 compromises human stromal cell support of hematopoietic progenitor cells or stimulates extramedullary hematopoiesis in humans. Additional studies will be necessary to establish whether endogenous FGF-2, when abnormally elevated, contributes to the pathogenesis of bone marrow failure and extramedullary hematopoiesis in patients who have clonal myeloid disorders.

Despite recent advances in the characterization of underlyng genetic lesions in clonal myeloid disorders and promising new therapies, most patients experience a recurrence of their disease. Ineffective hematopoiesis is a determinant of severe morbidity and a limiting factor for potentially curative therapies that target the malignant cells (1,5). We now provide evidence that defective bone marrow hematopoiesis, extramedullary hematopoiesis, and spleen-megaly in patients with clonal myeloid disorders may reflect the abnormally high levels of FGF-2 these patients display. Thus, FGF-2 represents a promising therapeutic target for correction of hematologic abnormalities of clonal myeloid disorders and, potentially, other diseases. Antibody neutralization and pharmacologic inhibition of activated tyrosine kinase receptors are effective treatment modalities (45) that could potentially be applied to FGF-2 and its receptors.

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**Notes**

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