Basic Fibroblast Growth Factor Antagonizes Transforming Growth Factor β-Mediated Erythroid Differentiation in K562 Cells

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Basic fibroblast growth factor (bFGF) and transforming growth factor-β1 (TGF-β) have both been shown to act on hematopoietic progenitor cells. bFGF is a hematopoietic cytokine that acts on progenitor cells in concert with other cytokines to promote their proliferation. TGF-β induces erythroid differentiation in K562 cells. To determine whether bFGF might act on progenitor cells by antagonizing the effects of cytokines that induce differentiation, we determined the effects of bFGF on the TGF-β–mediated induction of hemoglobin synthesis in K562 cells. bFGF antagonized the TGF-β–mediated induction of hemoglobin in a dose-dependent manner, with 0.1 ng/mL bFGF inhibiting hemoglobin induction by 40% and 10 ng/mL bFGF completely abrogating hemoglobin production. bFGF was most effective at antagonizing the TGF-β–mediated induction of hemoglobin if it and TGF-β were added simultaneously to K562 cells, but delayed addition of bFGF to TGF-β–treated cultures still resulted in significant inhibition of hemoglobin synthesis. The inhibitory effects of bFGF on hemoglobin production were fully reversible, showing that bFGF did not permanently alter the phenotype of K562 cells. The hemin-mediated induction of hemoglobin synthesis in K562 cells was only partially negated by bFGF. bFGF also diminished the expression of glycophorin A on the surface of K562 cells. These results indicate that bFGF might increase progenitor/stem cell numbers by antagonizing the effects of cytokines that induce differentiation, thereby increasing the pool of proliferating progenitor/stem cells.

BASIC FIBROBLAST growth factor (bFGF), a factor known to be involved in angiogenesis and wound healing,[1] is also a hematopoietic cytokine. It is produced by[2,3] and is a potent mitogen[4] for human bone marrow stromal cells. It also stimulates myelopoiesis in human long-term bone marrow cultures.[5] bFGF stimulates the growth of highly purified progenitor cells in concert with other hematopoietic cytokines such as interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), and stem cell factor.[6-8] There is evidence that bFGF promotes the proliferation of stem cells and maintains their primitive phenotype. Neuronal and primordial germ cells can be cultured indefinitely and maintain a stem cell phenotype in the presence of bFGF.[9-11] Huang and Terstappen[12] noted that single human fetal hematopoietic stem cells, cultured in the presence of bFGF and insulin-like growth factor 1, gave rise to both an adherent stromal cell layer and to primitive hematopoietic stem cells. bFGF has also been shown to repress the terminal differentiation of skeletal muscle cells[13,14] and in this manner it might also favor the proliferation of immature myoblasts.

We therefore determined whether bFGF might act on primitive hematopoietic cells to antagonize their differentiation and maintain their primitive phenotype. For these experiments, we used the human erythroleukemia cell line, K562,[15] that can be induced to undergo hemoglobinization by a number of chemical agents[16-18] and by transforming growth factor-β (TGF-β).[19] This line also expresses the red blood cell membrane protein glycophorin A,[20] which can be downregulated by exposing K562 cells to hexamethylene bisacetamide.[21] We found that bFGF prevented the TGF-β–mediated induction of hemoglobin in K562 cells, indicating that, in this system, bFGF antagonized the differentiation-inducing effects of TGF-β. bFGF also diminished the expression of glycophorin A on the surface of K562 cells. Thus, one of the functions of bFGF in vivo could be to promote a stem cell phenotype and antagonize the effects of cytokines that induce differentiation.

MATERIALS AND METHODS

Reagents. Recombinant human TGF-β1 was obtained from Genentech (South San Francisco, CA). Recombinant human bFGF was obtained from Synergen (Boulder, CO). Benzidine was obtained from Fluka AG (Buchs, Switzerland). Hemin was obtained from Sigma Chemical Co (St Louis, MO). Mouse antihuman glycophorin A and mouse IgG1 κ were obtained from Dako A/S (Glostrup, Denmark). Fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG was obtained from Cappel (West Chester, PA). Paraformaldehyde was obtained from BDH Chemicals Ltd (Poole, UK). [6-^{3}H] thymidine (1.0 mCi/mL) was obtained from Amersham International (Buckinghamshire, UK).

Methods. K562 cells were cultured in RPMI-1640 (Sigma) supplemented with 10% heat-inactivated fetal calf serum (FCS; State Vaccine Institute, Cape Town, South Africa) and antibiotics at 37°C in a humidified atmosphere with 5% CO_2 Cells at a density of 5×10^4 cells/mL or 1×10^5 cells/mL were incubated in media alone or media containing hemin, TGF-β (1 ng/mL), or bFGF at the indicated concentrations alone or in combination with TGF-β or hemin. Hemoglobin-containing cells were identified by staining with benzidine.[22]

For measuring DNA synthesis, K562 cells were seeded at 1×10^5 cells/mL in 200 μL of RPM1-1640 containing 10% FCS in media alone or media containing TGF-β (1 ng/mL) and/or bFGF at the indicated concentrations for 72 hours. ^{3}H-thymidine (0.2 μCi/well) was added to each well and cells were harvested and counted in a liquid scintillation counter.

Glycophorin A expression was determined on cells seeded at 2×10^5 cells/mL and cultured for 4 days in the absence or presence of bFGF (10 ng/mL). Cells (6×10^5) in 200 μL medium were incubated at 4°C for 30 minutes with 100 μL (10 μg IgG/mL) mouse antihuman glycophorin A. Cells were washed twice with 3 mL ice-

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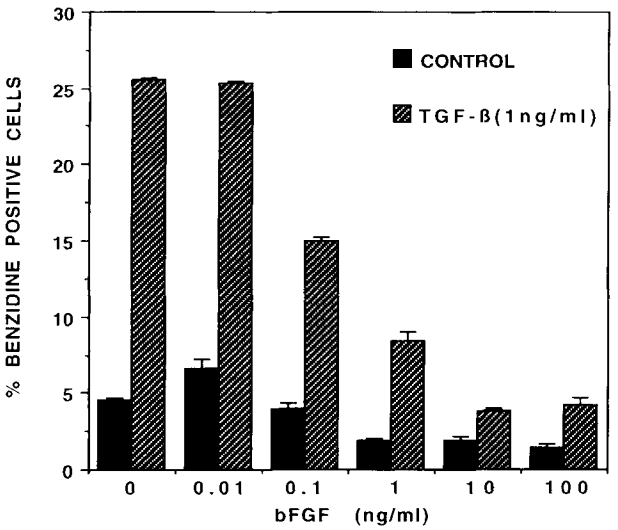

Fig 1. K562 cells (5 × 10⁴) were incubated (▨) with or (■) without TGF-β (1 ng/mL) and varying concentrations of bFGF as indicated. Cells were stained with benzidine for hemoglobin determination at day 4.

cold phosphate-buffered saline (PBS) to remove unbound antibody, resuspended in 100 μL FITC-labeled goat antimouse IgG (20 μg/mL), and incubated for 30 minutes at 4°C in the dark. The cells were then washed twice with 3 mL ice-cold PBS and resuspended in 300 μL 1% paraformaldehyde in PBS, pH 7.4. Mouse IgG1 was used as an isotype-matched negative control for each sample. Samples were analyzed by flow cytometry (EPICS Profile II; Coulter Corp, Hialeah, FL). Ten thousand events, occurring within the bitmap (forward scatter versus log side scatter), were analyzed for each sample. Voltages and scale factors were constant for each experiment. Histograms were smoothed (a weighted 3-point smooth was used) and overlayed using EPICS CytoLogic Software (Coulter Electronics).

RESULTS

Approximately 2% to 5% of untreated K562 cells contained hemoglobin. As noted by others,[19] TGF-β induced the synthesis of hemoglobin in K562 cells. Three days after the addition of 1 ng/mL TGF-β to cultures, 15% to 27% of K562 cells contained hemoglobin. Further incubation of K562 cells with TGF-β or additional amounts of TGF-β did not significantly increase the number of hemoglobin-containing cells.

The TGF-β–mediated induction of hemoglobin was antagonized by bFGF in a dose-dependent manner, with 0.1 ng/mL bFGF inhibiting hemoglobin induction by 40% and 10 or 100 ng/mL bFGF completely abrogating hemoglobin synthesis (Fig 1). bFGF alone at 1, 10, or 100 ng/mL consistently diminished the number of cells that endogenously produced hemoglobin (Fig 1).

bFGF did not have significant effects on the DNA synthesis (Fig 2) or growth of K562 cells (data not shown). Exposure of K562 cells to bFGF at 10 ng/mL for 3 days resulted in a 3% decrease in cell numbers (data not shown) and a 15% decrease in DNA synthesis relative to untreated K562 cells (Fig 2). TGF-β alone (1 ng/mL) modestly inhibited DNA synthesis, whereas more significant inhibition of DNA synthesis was noted when bFGF (1 to 100 ng/mL) and TGF-β (1 ng/mL) were both added to K562 cells (Fig 2). The reason for this is not known.

bFGF was most effective at antagonizing the TGF-β–mediated induction of hemoglobin if it and TGF-β were added simultaneously to K562 cells (Fig 3). Delayed addition of bFGF to TGF-β–treated cells partially abrogated the induction of hemoglobin in K562 cells. If bFGF was added 24, 48, or 72 hours after TGF-β addition, the inhibition of hemoglobinization was 69%, 30%, and 12%, respectively, indicating that substantial inhibition was still noted when bFGF was added to cells 24 hours after TGF-β addition and that partial inhibition was obtained when bFGF was added 48 hours after the addition of TGF-β.

The inhibitory effect of bFGF on the TGF-β–mediated hemoglobin production was reversible (Fig 4). If bFGF was added to K562 cells for 3 days, 1% of the cells contained hemoglobin (Fig 4A, column 3). Cells were washed free of reagents and incubated in the absence (Fig 4B) or presence (Fig 4C) of additional TGF-β for a further 3 days and the percentage of hemoglobin-containing cells was determined. Cultures that were treated with bFGF for 3 days, washed, and then incubated with TGF-β alone for an additional 3 days comprised 16% of the hemoglobin-containing cells (Fig 4C, column 3), compared with 17% found in the control TGF-β–treated population (Fig 4C, column 1). When TGF-β and bFGF were added simultaneously to cells for 3 days, 3% of the population contained hemoglobin. Removal of both reagents and subsequent addition of TGF-β alone for a further 3 days resulted in 14% of hemoglobin-containing cells, indicating that the effects of bFGF were readily reversible after its removal from the cultures.

Hemin has also been shown to induce hemoglobin production in K562 cells.[16,17] Interestingly, bFGF was much less effective at inhibiting hemin-induced hemoglobin production than TGF-β–induced hemoglobin synthesis. bFGF (10 ng/mL) inhibited hemoglobin induction by 10, 20, and 40 μmol/

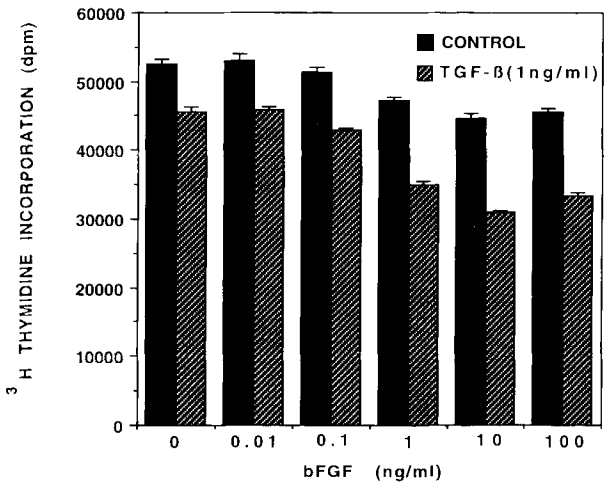

Fig 2. Two hundred microliters of K562 cells (10^5 cells/mL) was incubated (▨) with or (■) without TGF-β (1 ng/mL) and varying concentrations of bFGF as indicated for 72 hours. ^{3}H-thymidine (0.2 μCi/well) was added for the last 18 hours of the culture period.

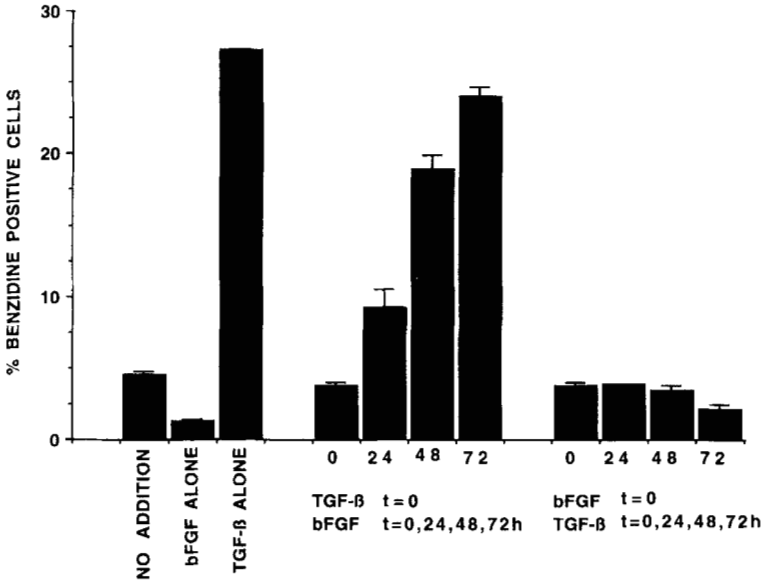

Fig 3. K562 cells (5 × 10^4) were incubated with no additions, with bFGF (10 ng/mL), or with TGF-β (1 ng/mL) for 4 days and benzidine-containing cells were scored. Four duplicate sets of cultures were prepared in which TGF-β was added at time 0 and bFGF was added at time 0 and after 24, 48, and 72 hours. In addition, 4 sets of cultures were prepared in which bFGF was added at time 0 and TGF-β was added at time 0 and after 24, 48, and 72 hours. All cells were examined for evidence of benzidine staining 4 days after inception of the experiment.

L hemin by 54%, 51%, and 14%, respectively (Fig 5). Thus, the hemin-mediated induction of hemoglobin synthesis could be inhibited by a maximum of 50% when lower concentrations of hemin were used as compared with the complete abrogation of the TGF-β–mediated induction of hemoglobin synthesis by bFGF. This indicates that the mechanisms by which TGF-β and hemin influence hemoglobin synthesis must differ.

bFGF also significantly downregulated the expression of glycophorin A on K562 cells (Fig 6). The number of glycophorin A-positive cells was reduced by approximately 18% and the mean fluorescence intensity was reduced by 41%. TGF-β (1 ng/mL) had no effect on glycophorin A expression on K562 cells.

DISCUSSION

We have shown that bFGF antagonizes the TGF-β–mediated induction of hemoglobin in K562 cells. The effects of bFGF are reversible, indicating that the continued presence of this factor is required to negate the differentiation-inducing effects of TGF-β and that cells treated with bFGF do not undergo an irreversible change in phenotype. The expression of glycophorin A on K562 cells was also downregulated by bFGF.

bFGF, in concert with IL-3 and GM-CSF has been shown to augment the cloning potential of isolated human bone marrow progenitor cells.[6,7,8,23] The addition of bFGF to human long-term bone marrow cultures results in increases of up to 100-fold in progenitor cells in the adherent cell layer.[5] The mechanism by which bFGF increases the progenitor cell content of these cultures is not known. A possible mechanism could be the abrogation of the effects of inhibitory cytokines such as TGF-β; bFGF has been shown to counteract partially the inhibitory effects of TGF-β on myeloid progenitor cell proliferation.[23]

The data presented in this manuscript indicate that bFGF could also increase progenitor cell numbers by antagonizing the effects of cytokines that induce differentiation. Recent

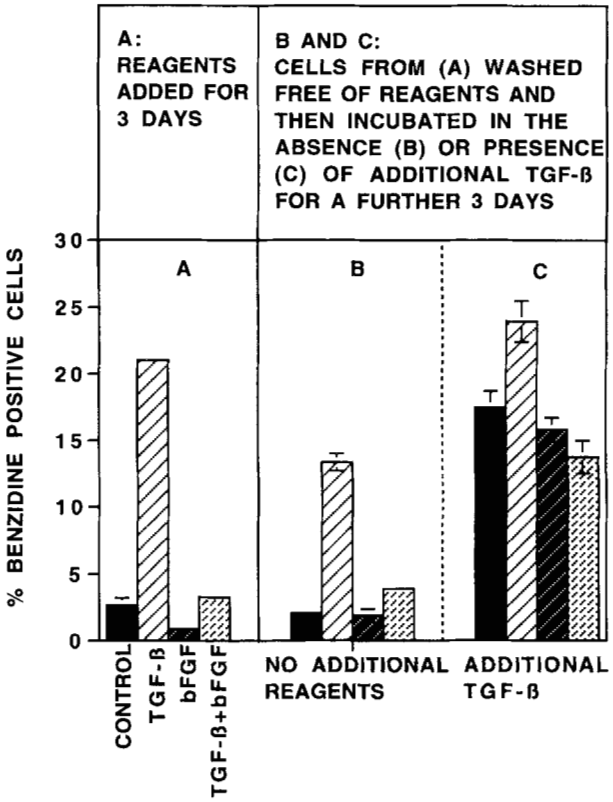

Fig 4. K562 cells (5 × 10^4) were incubated with no additions, with TGF-β (1 ng/mL), with bFGF (10 ng/mL), or with TGF-β + bFGF for 3 days (A). Cultures were washed free of reagents and then incubated for an additional 3 days in the absence (B) or presence of further TGF-β (1 ng/mL) (C). Benzidine-positive cells were determined.

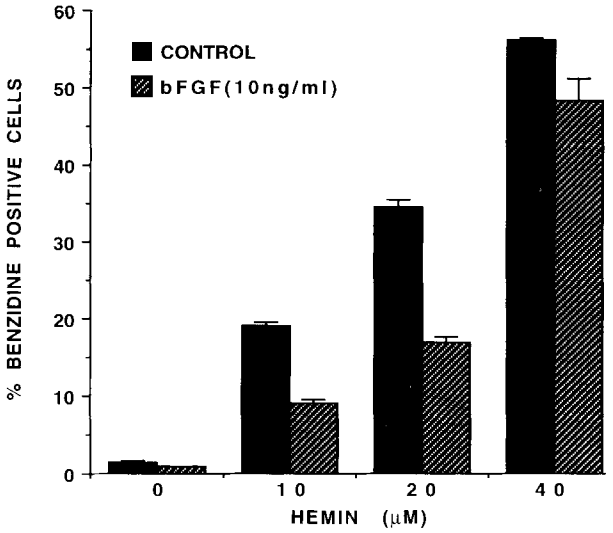

Fig 5. K562 cells (1 × 10^5) were incubated (▨) with or (■) without bFGF (10 ng/mL) and varying concentrations of hemin as indicated. Cells were stained with benzidine for hemoglobin determination at day 4.

data from Huang and Terstappen[12] support this notion. bFGF and insulin-like growth factor 1 added to single human fetal $CD34^+$ $CD38^-$ bone marrow cells induce the establishment of an adherent stromal cell layer containing primitive hematopoietic stem cells. If these cells are seeded in medium containing bFGF, insulin-like growth factor 1, IL-3, GM-CSF, IL-6, stem cell factor, and erythropoietin, only hematopoietic colonies were noted. Therefore, the hematopoietic growth factors prevented the proliferation of the subset of stem cells that gave rise to both the bone marrow microenvironment and the hematopoietic elements. This stem cell subset was only evident if cells were cultured with bFGF and insulin-like growth factor alone. Additional evidence that bFGF acts as a stem cell mitogen is provided by experiments with neuronal[9] and primordial germ cells[10,11] that indicate that bFGF maintains the primitive stem cell phenotype of these cells, permitting them to be cultured as cell lines.

It is possible that, in the bone marrow microenvironment, a balance exists between the concentration of stem cell factors, such as bFGF, that promotes stem cell renewal and differentiation-inducing factors that induce the lineage commitment of progenitor cells. bFGF is produced by human stromal cells.[2,3] There is evidence that specific cell lineages are localized to microcompartments in the bone marrow[24-26] and that the production of several cytokines (erythropoietin, IL-3, and granulocyte-CSF) is restricted to a small subset of bone marrow cells.[27-29] Experiments by Lowry et al[30] have indicated that stem cell factor produced at a local high concentration could act as an "anchor factor" in a hematopoietic niche, enabling the hematopoietic stem cells to respond to very low "subliminal" cytokine concentrations. Gradients of cytokines produced in the marrow microenvironments[31] could also dictate the lineage commitment of stem cells. It is therefore possible that bFGF produced by subsets of stromal or other hematopoietic cells[2] in niches, and present in high local concentrations, might favor proliferation of the "stem cell" phenotype and antagonize the influence of cytokines that promote differentiation. It is also possible that aberrant production of bFGF might result in excessive prolif-

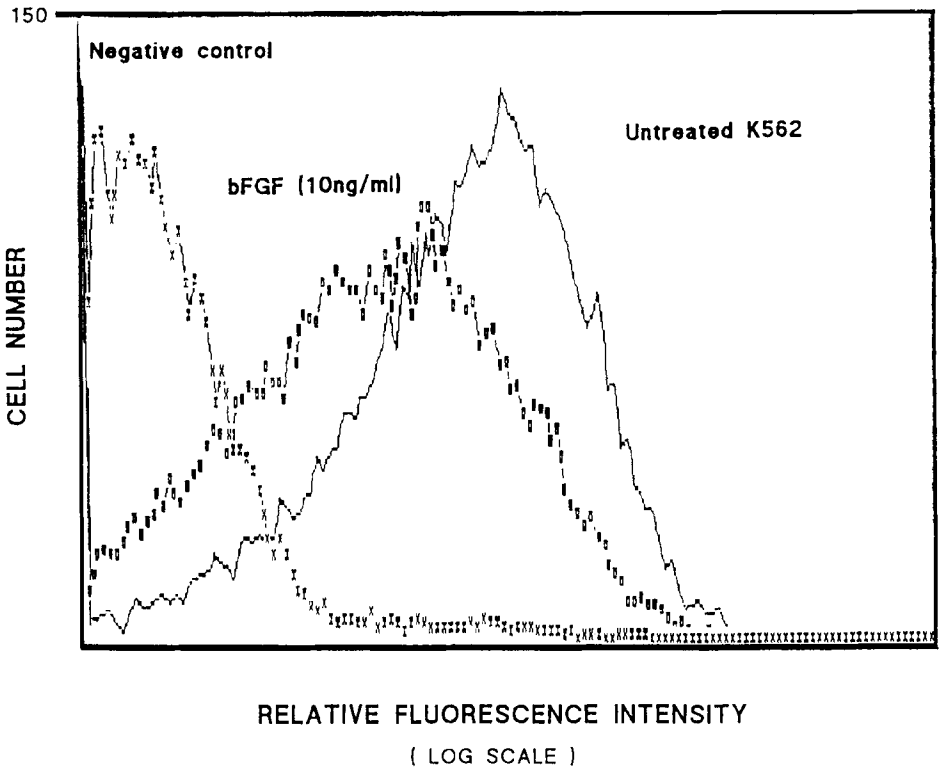

Fig 6. K562 cells were analyzed by flow cytometry for glycophorin A expression. (——) Untreated cells; (□ — □) bFGF (10 ng/mL); (X — X) isotype-matched negative control.

eration of stem cells, resulting in leukemias or myeloproliferative disorders.

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