Human Leukemia Cell Lines Bind Basic Fibroblast Growth Factor (FGF) on FGF Receptors and Heparan Sulfates: Downmodulation of FGF Receptors by Phorbol Ester

By John P. Liuzzo and David Moscatelli

Basic fibroblast growth factor (bFGF) has been identified as an important cytokine for blood cells. To determine whether hematopoietic cells have receptors that recognize bFGF, the ability of human leukemia cell lines to bind $^{125}$I-bFGF was investigated. Specific bFGF-binding sites were identified on K562 and HL60 cells, but not on U937 cells. DAlM cells bound low amounts of $^{125}$I-bFGF specifically. Binding of $^{125}$I-bFGF to K562 cell surfaces was reduced in a dose-dependent manner by unlabeled bFGF or by heparin. Scatchard analysis of binding to K562 cells revealed two classes of binding sites: 1,660 high affinity binding sites per cell with a dissociation constant (kd) of 192 pmol/L, and 38,600 low affinity sites per cell with a kd of 9.3 nmol/L. Chemical crosslinking experiments with K562, HL60, and DAlM cells revealed receptor-growth factor complexes with molecular masses of 140 to 160 kD, similar in size to complexes formed by known receptor species. Binding of $^{125}$I-bFGF to K562 cells was sensitive to heparinase treatment but not to chondroitinase treatment, suggesting that heparan sulfate proteoglycans (HSPGs) may be responsible for the low affinity binding sites. To further investigate whether K562 cells make HSPG, the incorporation of $^{35}$SO$_4$ into proteoglycans was assessed.

Metabolically labeled cell-surface proteoglycans with molecular masses of 180 to 300 kD were identified in K562 cells. These proteoglycans were sensitive to heparinase, demonstrating that K562 cells synthesize bFGF-binding HSPG. Treatment of K562 cells with phorbol-12-myristate-13-acetate (PMA) caused a loss of bFGF-binding capacity. This decreased binding capacity reflected a rapid loss of high affinity receptors. The ability to form bFGF-receptor complexes decreased by 65% to 70% within 1 hour and declined continuously thereafter. The decrease in binding of bFGF was not due to an autocrine downregulation of bFGF receptors, because there was no increase in bFGF after PMA treatment as detected by Western blotting, and suramin, which blocks bFGF binding to receptors, did not prevent the loss of receptors after exposure to PMA. In addition, inhibitors of either protein synthesis or protease activity did not prevent the loss of bFGF receptors in PMA-treated cells. In summary, this work demonstrates that leukemia cell lines have receptors that specifically bind bFGF and supports the hypothesis that bFGF acts directly on certain blood cells to stimulate their proliferation.

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A lower affinity (kd = 2 x 10$^{-9}$ mol/L), large capacity class of binding sites for bFGF has also been identified. These low affinity receptors are heparan sulfate proteoglycans (HSPGs) found on the cell surface and in the extracellular matrix. Basic FGF can be released from the HSPGs by enzymatic degradation of the glycosaminoglycan with heparanase or of the HSPG core protein with plasmin. In addition, phospholipid-anchored HSPGs can be cleaved by phospholipase C, releasing a biologically active bFGF-HSPG complex. HSPG-bound bFGF can also be released by an excess of soluble heparin or heparan sulfate. Both cell-associated and soluble heparan sulfates potentiate the binding of bFGF to its high affinity receptors.

A role for bFGF in hematopoiesis has recently become evident, but the mechanism has not been fully elucidated. Basic FGF is a potent mitogen for passaged bone marrow stromal cells and considerably delays their senescence. In addition, low concentrations of bFGF (0.2 to 2.0 ng/mL) induce myelopoiesis in long-term bone marrow culture. It...
is possible that bFGF acts indirectly by stimulating bone
marrow stromal cells to secrete signaling molecules that
stimulate the hematopoietic stem cells to proliferate. Ho-
ever, increasing evidence shows that bFGF may also act
directly to stimulate blood cell proliferation. Purified popu-
lations of human hematopoietic stem cells have been shown
to respond to bFGF with increased survival and proliferative
ability, suggesting a permissive role for bFGF in hematopo-
ietic colony formation. Therefore, bFGF produced by
bone marrow stromal cells could act through direct interac-
tions with FGFRs on the surfaces of blood cells, thereby
stimulating their proliferation and function.

To determine whether bFGF can interact directly with
blood cells, we have investigated the presence of functional
bFGF receptors on blood cell-derived cell lines. We have
found that the K562 human erythroleukemia, DAMI hu-
man megakaryoblastic leukemia, and HL60 human promy-
elocytic leukemia52 cell lines bind bFGF on typical trans-
membrane protein receptors. In addition, K562 cells express
HSPGs that can bind bFGF and, therefore, may be involved
in modulation of its biologic activity. We have also found
that the bFGF receptors can be modulated after treatment of
K562 cells with phorbol ester.

MATERIALS AND METHODS

Cell culture. K562, DAMI, HL60, and U937 cells were grown
in suspension using RPMI 1640 medium supplemented with 10%
feetal calf serum (Intergen, Purchase, NY), 100 U/mL penicillin,
and 100 µg/mL streptomycin in a humid atmosphere with 5% CO2
at 37°C. In some experiments, the K562, DAMI, U937, or HL60 cells
were treated for varying lengths of time with 10-8 mol/L phorbol
12-myristate-13-acetate (PMA; Sigma, St Louis, MO), which has
been used to induce differentiation of these cells.53-55 The cells
were harvested after PMA treatment using Ca2+ and Mg2+-free phosphate-
buffered saline (PBS) containing 10 mmol/L EDTA and a sterile
rubber cell scraper to detach the cells from the plastic dishes.

125I labeling of bFGF and aFGF. Recombinant human bFGF
was a gift from Synergen, Inc (Boulder, CO) and was labeled with
1 mCi Na2125I (NEN, Boston, MA) using the Iodo-Gen procedure
(Pierce Chemical Co, Rockford, IL) as described previously.17 Ra-
diolabeled bFGF was separated from unincorporated 125I on a G-25
Sepharose column (Pharmacia, Piscataway, NJ). The specific activity
was 1,400 to 2,800 cpm/µmol.

Recombinant human αFGF was provided by Dr J. Schlessinger
of New York University Medical Center (New York, NY) and was labeled with 125I using chloramine T (Eastman Kodak Co, Rochester,
NY), as described.18 Radiolabeled αFGF was separated from unincor-
porated 125I by affinity chromatography on heparin-Sepharose (Phar-
macia). The specific activity was 300 to 600 cpm/µmol.

Analysis of iodinated bFGF or αFGF by sodium dodecyl sulfate-
polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiog-
raphy revealed a single band migrating at the appropriate position.
The recovery of bFGF or αFGF after iodination was determined by
competition binding assays. CHO-DG44 cells expressing FGFR-
12 were incubated for 2 hours at 4°C with an amount of radiolabeled
bFGF or αFGF sufficient to saturate high affinity binding sites and
varying concentrations of unlabeled ligand. The amount of radiola-
beled bFGF bound to receptors was determined as described.18 The
concentration of radiolabeled bFGF or αFGF was considered to be
equal to the concentration of unlabeled ligand that inhibited binding
of labeled ligand to receptors by 50%. In these experiments, nonspe-
cific binding was less than 10% of the total binding (see Table 1).

Greater than 80% of the biologic activity of bFGF was recovered
after iodination.

Binding of 125I-bFGF or 125I-αFGF to leukemia cells. Cells
grown in suspension were collected and counted with a hemocytom-
eter for all experiments. The cells were resuspended at 2.5 × 107/
mL in serum-free RPMI medium containing 0.15% gelatin, 25 mmol/
L HEPES pH 7.5, and 10 ng/mL 125I-bFGF or 125I-αFGF. This
concentration of bFGF was sufficient to saturate receptors, as experi-
ments with other cell types have shown.17,56 In some experiments,
the gelatin in the binding medium was replaced by bovine serum
albumin (BSA), and identical results were obtained. For each sample,
1 mL of the cell suspension in a 1.5-mL Eppendorf tube was incu-
ated on an end-over-end mixer at 4°C for at least 2 hours to achieve
equilibrium between bFGF and receptors. At the end of the incuba-
tion, the cells were washed two times with cold PBS. After each
wash, the cells were collected by centrifugation, and the wash fluid
was aspirated. After the last wash, the bottoms were cut from the
Eppendorf tubes and counted for gamma emission for 1 minute each.
All experiments were performed in either duplicate or triplicate.

In some experiments, CHO-DG44 cells expressing FGFR-1 were
used as controls. These cells express 100,000 FGFRs per cell as
determined by Scatchard analysis of 125I-bFGF binding data.17 The
CHO/FGFR-I cells also produce bFGF-binding HSPGs.57 With these
cells, salt washes were used to separate radiolabeled bFGF bound to
HSPGs from that bound to receptors.57 CHO/FGFR-I cells that had been
incubated with 125I-bFGF were washed twice with PBS to remove
unbound ligand, twice with 2 mol/L NaCl in 20 mmol/L HEPES pH 7.4
to remove HSPG-bound ligand, and three times with 2 mol/L NaCl in
20 mmol/L sodium acetate pH 4.0 to remove FGFR-
bound ligand.

Crosslinking experiments. To identify FGFRs, complexes be-
 tween 125I-bFGF or 125I-αFGF and receptor were crosslinked, extracts
of the cells were run on SDS-polyacrylamide gels, and the complexes
were detected by autoradiography. Briefly, the cells (5 × 105 or 10
× 105 cells per condition) were incubated with 125I-bFGF or
125I-αFGF at 4°C as described above. After two washes with PBS,
cells were resuspended in 1 mL PBS containing 1 mmol/L homobifunc-
tional crosslinking agent, bis(sulfosuccinimidyl)suberate (BS3;
Pierce Chemical Co), and incubated at room temperature for 30 to
60 minutes on an end-over-end mixer. The reaction was quenched
by addition of 50 mmol/L glycine, and after 5 minutes, the cells
were collected by centrifugation, washed twice with PBS, and
extracted in 1% Triton X-100 containing 2% glycerol, 0.1 mg/mL
aprotinin, 0.1 mg/mL leupeptin, and 20 mmol/L EDTA. Extracts
were incubated on ice for 10 to 20 minutes before centrifugation at
16,000g for 1 minute to pellet insoluble material. The supernatants
were mixed with SDS-PAGE reducing sample buffer, heated in a
boiling water bath for 2 minutes, and loaded on an SDS-polyacry-
lamide gel. The gels were composed of 5% to 15% gradient resolv-
ing gel, and the complexes were detected by autoradiography. Briefly,
the bands were exposed to phosphorimager screens (Molecular Dyna-
tics, Sunnyvale, CA) for 2 days or exposed to XAR-5 film (Eastman Kodak)
for 2 to 3 weeks.

Heparinase or chondroitinase treatment of K562 cells. K562
cells (20 × 107) were incubated with 5 U/mL Flavobacterium hep-
rinum heparinase or 0.02 U/mL Proteus vulgaris chondroitinase
ABC (Sigma) in PBS containing 0.1% BSA for 2 to 4 hours at 25°C.
Control cells were either untreated or incubated in PBS alone at
room temperature for the same period. Cells were collected by cen-
trifugation and washed twice with PBS. The treated cells were
divided into equal aliquots and were used in binding or crosslinking
experiments as described above.

15SO4 labeling of K562 cells. K562 or HL60 cells were grown in
normal culture medium containing 40 µCi/mL sodium 15SO4 (NEN).
After a 24-hour incubation at 37°C, the cells were collected, washed

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FGF RECEPTORS IN LEUKEMIA CELLS

The ability of the leukemia cell lines K562, DAMI, HL60, and U937 to bind 125I-bFGF was measured. Cells in suspension were incubated at 4°C in medium containing 10 ng/mL 125I-bFGF, and after 2 hours, the amount of 125I-bFGF bound was determined. K562 and HL60 cells bound significant amounts of 125I-bFGF. Approximately 50% of the binding was competed by inclusion of 100-fold excess unlabeled bFGF. An excess of unlabeled bFGF competed poorly for binding, reducing the amount of 125I-bFGF bound to these cells by 23%. U937 cells bound little 125I-bFGF, and excess unlabeled bFGF did not reduce the amount bound. As bFGF binds to both receptors and HSPGs on cells and soluble heparin can compete for binding to HSPGs,17 the effect of soluble heparin on the binding of 125I-bFGF to leukemia cell lines was determined. Heparin reduced the binding of 125I-bFGF to K562 and HL60 cells by approximately 66% and 68%, respectively (Fig 1). Similarly, 10 μg/mL heparin competed for binding of 125I-bFGF to DAMI cells, reducing binding by 27%. Heparin had no effect on the already low basal levels of 125I-bFGF binding to U937 cells. The combination of 10 μg/mL heparin and excess unlabeled bFGF reduced binding of 125I-bFGF slightly more than either heparin or unlabeled bFGF alone. Thus, bFGF binds to sites on K562 and HL60 cells that can be inhibited both by excess unlabeled bFGF and by heparin. The remaining 125I-bFGF bound in the presence of both soluble heparin and excess unlabeled bFGF probably represents nonspecific binding. This nonspecific binding represents 25% of the total binding to K562 cells. The nonspecific binding appears high compared with total binding, but the amount of nonspecific binding per cell is comparable with the nonspecific binding detected in Chinese hamster ovary (CHO) cells expressing transfected receptors (see Table 1).

The effects of heparin and excess bFGF on binding of 125I-bFGF to K562 cells and HL60 cells were compared with effects on CHO cells expressing transfected FGF-R-1 (CHO/FGFR-1 cells). In the CHO/FGFR-1 cells, binding to HSPGs can be separated from binding to receptors by a salt wash.17 Attempts to use these procedures with K562 and HL60 cells were unsuccessful because the high salt buffers damaged the cells. Table 1 shows that addition of 10 μg/mL soluble heparin inhibited binding of 125I-bFGF to HSPGs on the CHO/FGFR-1 cells by 80% but had little effect on binding to receptors, causing only a 17% reduction. In contrast, unlabeled bFGF inhibited 92% of the binding of 125I-bFGF to receptors on the CHO/FGFR-1 cells but only 49% of the binding to HSPGs. Binding of 125I-bFGF to K562 and HL60 cells was inhibited by both heparin and unlabeled bFGF. As binding to receptors is relatively resistant to competition by heparin, these results suggested that a component of the binding to these cells is due to HSPGs.

The effect of addition of unlabeled bFGF on binding of 125I-bFGF to K562 cells was investigated further. As shown in Fig 2A, maximum inhibition of 125I-bFGF binding to K562 cells was obtained with 2 μg/mL bFGF, a 200-fold excess.
effect, so that approximately 36% of the *I-bFGF bound maximal inhibition was obtained with 10 pg/mL heparin. Higher concentrations (1 to 2 ng/mL bFGF was observed with as little as 20 ng/mL heparin, and concentrations of heparin were included in the binding experiment indicated the presence of two types of binding sites for bFGF. This could be detected in the presence of 1 pg/mL unlabeled bFGF, and maximal inhibition was obtained with 10 \( \mu \text{g/mL} \) heparin. Addition of higher concentrations of heparin had no further effect, so that approximately 36% of the 125I-bFGF bound was not inhibited by heparin. Although heparin potentiates bFGF binding to FGFRs on cells lacking heparan sulfates, heparin did not increase the level of 125I-bFGF binding at any concentration in these experiments.

The competition by unlabeled bFGF appeared biphasic. Low concentrations of unlabeled bFGF (50 to 100 ng/mL, a 5- to 10-fold excess) competed for 22% of the binding, intermediate concentrations did not decrease binding further, and higher concentrations (1 to 2 \( \mu \text{g/mL} \), a 100- to 200-fold excess) competed for another 35% of the binding. Concentrations of bFGF up to 10 \( \mu \text{g/mL} \) did not result in any further competition. The biphasic competition observed in this experiment indicated the presence of two types of binding sites with differing affinities for bFGF.

The effect of heparin on the binding of 125I-bFGF to K562 cells was also examined in more detail (Fig 2B). If varying concentrations of heparin were included in the binding medium with 10 ng/mL 125I-bFGF, inhibition of binding of 125I-bFGF was observed with as little as 20 ng/mL heparin, and maximal inhibition was obtained with 10 \( \mu \text{g/mL} \) heparin. Addition of higher concentrations of heparin had no further effect, so that approximately 36% of the 125I-bFGF bound was not inhibited by heparin. Although heparin potentiates bFGF binding to FGFRs on cells lacking heparan sulfates, heparin did not increase the level of 125I-bFGF binding at any concentration in these experiments.

As the competition with unlabeled bFGF indicated two classes of binding sites for bFGF on K562 cells, the presence of multiple sites was investigated by Scatchard analysis. The binding data obtained when K562 cells were incubated with varying concentrations of 125I-bFGF gave a curve with two components (Fig 3) when plotted according to Scatchard, indicating the presence of two classes of binding site. The higher affinity component of the curve indicated 1,650 sites per cell, with a kd of 1.92 \( \times 10^{-10} \text{ mol/L} \). Extrapolation of the second component of the curve gave an estimate of 37,600 sites per cell, with a kd of 9.34 \( \times 10^{-7} \text{ mol/L} \). Thus, the K562 cells express approximately 23 times more low affinity binding sites than high affinity binding sites. The affinity for the low affinity sites is in the same range as the affinity of 125I-bFGF for heparan sulfates on other cells. These observations are consistent with the fact that heparin can compete effectively for binding of 125I-bFGF to K562 cells.

To determine if specific protein bFGF receptors are present on leukemia cells, complexes of 125I-bFGF with receptor were crosslinked and visualized by autoradiography after separation of proteins by SDS-PAGE. K562 and HL60 cells displayed receptor complexes with molecular weights of 140 to 160 kD, similar to the size previously reported for frizzled receptors. However, K562, DAMI, and HL60 cells displayed only low levels of specific binding in Fig 1, and the receptor complexes could not be detected in U937 cells, even with very long exposures (data not shown). In K562, DAMI, and HL60 cells, the crosslinked bands were eliminated when incubation of the cells with 125I-bFGF occurred in the presence of 1 \( \mu \text{g/mL} \) unlabeled bFGF, demonstrating that the interaction was specific (Fig 4A, b lanes). The intensity of the receptor complex in DAMI cells was lower and was only visible after long film exposure, consistent with the low levels of specific binding found on these cells. The crosslinked receptor complexes appeared as multiple bands in each of these three cell types. In contrast, crosslinked 125I-bFGF-receptor complexes also could be detected in these cells (Fig 4A). The intensity of the receptor complex in DAMI cells was low and was only visible after long film exposure, consistent with the low levels of specific binding found on these cells. The crosslinked receptor complexes appeared as multiple bands in each of these three cell types. In contrast, crosslinked 125I-bFGF-receptor complexes could not be detected in U937 cells, even with very long exposures (data not shown). In K562, DAMI, and HL60 cells, the crosslinked bands were eliminated when incubation of the cells with 125I-bFGF occurred in the presence of 1 \( \mu \text{g/mL} \) unlabeled bFGF, demonstrating that the interaction was specific (Fig 4A, b lanes). Heparin at 1 or 10 \( \mu \text{g/mL} \) reduced the formation of crosslinked complex by 50% or 75%, respectively (data not shown, and Fig 4A, c lanes). Thus, high concentrations of heparin can inhibit binding to receptors in addition to blocking the binding to HSPGs in K562 cells. In addition to the complexes of 140 to 160 kD, specific radiolabeled complexes with molecular weights greater than 215 kD were also formed in K562, DAMI, and HL60 cells. These bands may represent dimers of the receptors.

Because bFGF binds to some FGFRs with lower affinity than others, it is possible that some FGFRs might be missed using 125I-bFGF as a probe. Because aFGF binds to all known FGFRs with high affinity, the binding of 125I-aFGF was compared with the binding of 125I-bFGF in K562 cells. K562 cells were incubated for 2 hours at 4°C with 10 ng/mL 125I-aFGF, and complexes of 125I-aFGF with receptor were cross-linked and visualized by autoradiography after separation of proteins by SDS-PAGE. aFGF was observed with as little as 20 ng/mL heparin, and maximal inhibition was obtained with 10 \( \mu \text{g/mL} \) heparin. Addition of higher concentrations of heparin had no further effect, so that approximately 36% of the 125I-bFGF bound was not inhibited by heparin. Although heparin potentiates bFGF binding to FGFRs on cells lacking heparan sulfates, heparin did not increase the level of 125I-bFGF binding at any concentration in these experiments.

### Table 1: Human Leukemia Cell Lines Bind aFGF and bFGF

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>125I-Ligand</th>
<th>No Competition</th>
<th>+aFGF</th>
<th>+bFGF</th>
<th>+Heparin</th>
</tr>
</thead>
<tbody>
<tr>
<td>K562</td>
<td>aFGF</td>
<td>3.0 ± 0.2</td>
<td>2.1 ± 0.1</td>
<td>1.4 ± 0.2</td>
<td>1.2 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>bFGF</td>
<td>21.2 ± 0.8</td>
<td>19.2 ± 2.1</td>
<td>9.1 ± 1.4</td>
<td>11.4 ± 0.8</td>
</tr>
<tr>
<td>HL60</td>
<td>aFGF</td>
<td>6.2 ± 0.5</td>
<td>3.4 ± 0.3</td>
<td>2.6 ± 0.4</td>
<td>5.5 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>bFGF</td>
<td>28.9 ± 2.2</td>
<td>14.2 ± 2.7</td>
<td>12.9 ± 1.3</td>
<td>7.4 ± 0.6</td>
</tr>
<tr>
<td>CHO/FGFR1 (receptors)</td>
<td>aFGF</td>
<td>85.7 ± 6.0</td>
<td>4.0 ± 0.3</td>
<td>ND</td>
<td>70.8 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>bFGF</td>
<td>54.7 ± 3.7</td>
<td>ND</td>
<td>4.6 ± 0.4</td>
<td>45.4 ± 11.1</td>
</tr>
<tr>
<td>CHO/FGFR1 (heparan sulfates)</td>
<td>aFGF</td>
<td>39.3 ± 1.8</td>
<td>25.1 ± 2.3</td>
<td>ND</td>
<td>27.9 ± 2.3</td>
</tr>
<tr>
<td></td>
<td>bFGF</td>
<td>38.9 ± 5.4</td>
<td>ND</td>
<td>20.0 ± 2.9</td>
<td>7.6 ± 0.3</td>
</tr>
</tbody>
</table>

K562 and HL60 cells were incubated for 2 hours at 4°C in serum-free medium containing 0.15% BSA, 25 mmol/L HEPES (pH 7.0), 10 ng/mL 125I-aFGF or 125I-bFGF, 0 or 1 \( \mu \text{g/mL} \) unlabeled aFGF or bFGF, and 0 or 10 \( \mu \text{g/mL} \) heparin. For comparison, transfected CHO cells expressing FGFR-1 were incubated with the same ligands under identical conditions. Leukemia cells were washed twice with PBS to remove unbound ligand, and total cell-bound radioactivity was measured. CHO/FGFR-1 cells were washed twice with PBS to remove unbound ligand, washed twice with 2 mol/L NaCl in 20 mmol/L HEPES pH 7.4 to remove heparan sulfate-bound ligand, and washed three times with 2 mol/L NaCl in 20 mmol/L sodium acetate, pH 4.0, to remove FGFR-bound ligand. For CHO/FGFR-1 cells, the results from the two washes are presented separately. Results are presented as femtomoles of iodinated growth factor bound per 10⁶ cells and are the averages of duplicate determinations with calculated standard deviations.

Abbreviation: ND, not determined.
FGF receptors in leukemia cells

Fig 2. Dose-dependent competition of 125I-bFGF binding in K562 cells. The human leukemia cell line, K562, was incubated with 10 ng/mL 125I-bFGF in serum-free medium containing 0.15% gelatin and 25 mM HEPES, pH 7.0, for 2 hours at 4°C in the presence of the indicated concentrations of (A) unlabeled bFGF or (B) heparin. Cells were washed twice with PBS to remove non-bound ligand, and cell-bound radioactivity was measured. Each point represents the average of duplicate determinations with calculated standard deviation.

Linked and visualized by autoradiography after separation of proteins by SDS-PAGE (Fig 4B). Receptor complexes crosslinked to 125I-aFGF were similar in size to the complexes observed with bFGF, with molecular weights of 140 to 160 kD. The formation of receptor complexes with 125I-aFGF could be prevented by inclusion of a 100-fold excess of either unlabeled aFGF or bFGF (Fig 4B). Similarly, formation of receptor complexes with 125I-bFGF could be prevented by a 100-fold excess of unlabeled aFGF (data not shown), demonstrating that the two ligands recognize the same receptors in K562 cells. When the amount of 125I-aFGF bound by K562 cells was quantitated, only low amounts of specific binding could be observed (Table 1). Indeed, K562 cells bound less 125I-aFGF than 125I-bFGF on CHO cells expressing FGFR-1 (Table 1). The small amount of 125I-aFGF compared with 125I-bFGF bound to K562 cells probably reflects the low number of receptors expressed on these cells and the binding of 125I-bFGF to HSPGs.

Basic FGF has been reported to block the stimulation of hemoglobin synthesis in response to transforming growth factor (TGF)-β in K562 cells. To verify that the bFGF receptors identified in our K562 cells were functional, we investigated whether bFGF would inhibit hemoglobin synthesis in response to TGF-β treatment. As reported by Burger et al., treatment of the cells with 1 ng/mL TGF-β for 72 hours resulted in 11.4% ± 1.8% of the K562 cells staining with benzidine, indicating the presence of hemoglobin, whereas only 3.4% ± 1.1% of untreated cells stained. Addition of 10 ng/mL bFGF with the TGF-β antagonized the induction of hemoglobin, and only 2.1% ± 1.5% of the cells were stained with benzidine (data not shown). Thus, the bFGF receptors identified in these K562 cells were able to transmit a signal that blocked the TGF-β induction of benzidine staining.

bFGF-binding HSPGs are present on the surface of K562 cells. To test whether part of the 125I-bFGF binding in K562 cells was due to the presence of HSPGs, cells were treated with heparinase or chondroitinase ABC and then were assayed for their ability to bind 125I-bFGF. Treatment with chondroitinase did not affect the binding of 125I-bFGF to K562 cells (data not shown). However, treatment with heparinase for 2 hours reduced the binding of 125I-bFGF by 75% (Fig 5A). Inclusion of 100-fold excess unlabeled bFGF or 10 µg/mL heparin along with the 125I-bFGF caused no further

Fig 3. Scatchard analysis of 125I-bFGF binding to K562 cells reveals two types of binding sites. K562 cells (2.5 × 10⁶ cells per point) were incubated for 2 hours at 4°C in serum-free medium containing 0.15% gelatin, 25 mM HEPES pH 7.0, and a range of concentrations of 125I-bFGF from 0.1 ng/mL to 40 ng/mL. Each concentration was assayed in duplicate. Cells were washed twice with PBS to remove non-bound ligand, and cell-bound radioactivity was measured. Non-specific binding was estimated by parallel determinations in which 1 ng/mL unlabeled bFGF was included. The results were plotted according to the method of Scatchard. The data are best described by two straight lines representing two binding sites.
Fig 4. Basic FGFRs in human leukemia cell lines. (A) K562, DAM1, and HL60 cells were incubated with 10 ng/mL $^{125}$I-bFGF for 2 hours at 4°C as described in the legend to Fig 1. The cells were collected by centrifugation, resuspended in PBS containing the crosslinking agent BS$_2$ (1 mmol/L), and incubated for 30 minutes at room temperature. Glycine (50 mmol/L) was added to quench the crosslinking reaction, and cells were washed once with PBS and extracted. Samples were analyzed under reducing conditions on a 3% to 15% gradient SDS-PAGE gel. Each lane contains lysate from 5 x 10$^6$ cells. Lanes a, $^{125}$I-bFGF alone; lanes b, $^{125}$I-bFGF with 1 µg/mL unlabeled bFGF; lanes c, $^{125}$I-bFGF with 10 µg/mL heparin; lanes d, $^{125}$I-bFGF with both 1 µg/mL unlabeled bFGF and 10 µg/mL heparin. Longer autoradiographic exposure was required for visualization of the receptor bands in DAM1 and HL60 cells. (B) K562 cells were incubated with 10 ng/mL $^{125}$I-aFGF for 2 hours at 4°C, and ligand-receptor complexes were crosslinked as described in A. Lane a, $^{125}$I-aFGF alone; lane b, $^{125}$I-aFGF with 1 µg/mL unlabeled aFGF; lane c, $^{125}$I-aFGF with 10 µg/mL heparin; lane d, $^{125}$I-aFGF with 1 µg/mL bFGF.

A reduction in binding of $^{125}$I-bFGF to K562 cells treated with heparinase, suggesting that the residual 25% of $^{125}$I-bFGF binding was nonspecific. The reduction in binding of $^{125}$I-bFGF to heparinase-treated K562 cells was similar to the reduction obtained in untreated cells in the presence of 10 µg/mL heparin. To determine whether binding to FGFRs on these cells is sensitive to heparinase, the formation of complexes between receptor and $^{125}$I-bFGF was investigated in heparinase-treated cells. Cells preincubated with heparinase or chondroitinase for 4 hours were incubated for 2 hours at 4°C with $^{125}$I-bFGF, and receptor-bFGF complexes were chemically crosslinked. In heparinase-treated cells, there was a 50% reduction in intensity of the $^{125}$I-bFGF–receptor complex (Fig 5B). In contrast, cells pretreated with chondroitinase displayed crosslinked receptor complexes equal in intensity to untreated cells (Fig 5B). As it has been reported that heparan sulfates or heparin is necessary for binding of bFGF to receptors, the ability of heparin to restore $^{125}$I-bFGF binding to heparinase-treated cells was investigated. However, addition of 0.1 to 10 µg/mL heparin to heparinase-treated cells did not increase formation of $^{125}$I-bFGF–receptor complexes (Fig 5B). Furthermore, if cells were first crosslinked to $^{125}$I-bFGF and subsequently treated with heparinase for 4 hours, $^{125}$I-bFGF–receptor complex bands were not reduced in size or intensity, demonstrating that HSPGs do not form a significant part of the crosslinked complex (data not shown). The finding that specific binding of $^{125}$I-bFGF to K562 cells was almost eliminated by heparinase treatment but that formation of $^{125}$I-bFGF–receptor complexes was only partially reduced suggests that the majority of the binding measured in these cells is due to HSPGs. This conclusion is supported by the results of the Scatchard analysis (Fig 3).

To demonstrate that cell surface HSPGs are present on K562 cells, proteoglycans were labeled by metabolic incorporation of $^{35}$SO$_4$. When extracts of these cells were run on SDS-PAGE and the gels were exposed for autoradiography, a broad $^{35}$SO$_4$-labeled band with molecular weights of 180 to 300 kD was observed (Fig 6, lane a). The intensity of the diffuse $^{35}$SO$_4$-labeled band was reduced almost totally after treatment of the intact cells with heparinase (Fig 6, lane b). Chondroitinase treatment of $^{35}$SO$_4$-labeled cells had little effect on the intensity of this band (Fig 6, lane c). Treatment with both enzymes completely eliminated the $^{35}$SO$_4$-labeled band (data not shown). These results show that K562 cells produce cell-surface proteoglycans that contain heparan sulfate. The decreased intensity of the bands after treatment of intact cells with heparinase suggests that the HSPGs are on the cell surface rather than sequestered in an intracellular compartment. Thus, K562 cells express cell-surface HSPGs that may be responsible for the low affinity bFGF binding sites on these cells.

PMA causes rapid downmodulation of bFGF receptors. Treatment for 24 to 48 hours with the protein kinase C (PKC)
activator PMA will cause megakaryocytic differentiation of K562 cells. The effect of PMA treatment on FGFR expression in K562 cells was determined. Addition of 10^{-8} mol/L PMA to cultures of K562 cells resulted in a reduction in their ability to bind 125I-bFGF (Fig 7A). Specific binding of 125I-bFGF was decreased by 35% after 1 hour, 41% after 2 hours, 53% after 6 hours, and 82% after 12 hours. Nonspecific binding was not significantly affected by PMA treatment. The ability to form 125I-bFGF–receptor complexes was even more markedly affected by PMA treatment (Fig 7B, lanes a to e). The intensity of the crosslinked bands formed was reduced by 69% in 1 hour, 82% in 2 hours, 93% in 6 hours, and 98% in 12 hours (Fig 7C). Thus, treatment with PMA causes a decrease in functional FGFRs with a half-time of less than 1 hour. Treatment of K562 cells with 10^{-8} mol/L 4alpha-phorbol, which does not activate PKC, or the vehicle dimethyl sulfoxide (DMSO) alone did not affect 125I-bFGF binding to its receptor (data not shown). Therefore, the downmodulation of FGFRs by PMA can be ascribed to its activation of PKC.

PMA treatment of K562 cells has been shown to induce expression of TGF-β, and it is possible that the reduction of receptors in response to PMA is mediated by TGF-β. However, 2- and 6-hour treatments with TGF-β had no effect on receptor complexes (Fig 7B, lanes h and i). PMA has also been reported to increase expression of bFGF in dermal fibroblasts. To investigate the possibility that PMA increased the expression of bFGF, resulting in autocrine downregulation of the FGFR, the effect of PMA on bFGF levels in K562 cells was investigated. Western blotting of K562 lysates with purified anti-bFGF antibodies showed no alterations in the amount of bFGF protein in PMA-treated cells (data not shown). Therefore, the changes in FGFR are not correlated with changes in bFGF expression. Moreover, the addition to the culture medium of 1 mmol/L suramin, which blocks the interaction of bFGF with receptors, or 10 μg/mL heparin, which competes for most of bFGF binding in K562 cells, did not prevent the decrease in FGFRs in PMA-treated cells (Fig 7B, lanes f and g). These results indicate that the decrease in FGFRs in PMA-treated cells is not the result of autocrine downregulation of receptors. In addition, inhibition of protein synthesis with cycloheximide did not prevent modulation of FGFRs by PMA and did not cause receptor downmodulation in the absence of PMA. These data suggest that PMA-induced FGF downmodulation does not require new protein synthesis, nor does PMA act by inhibiting the synthesis of FGFRs.

PMA treatment of cells has been shown to result in cleavage of the extracellular ligand-binding domain of some growth factor receptors, and differentiation-related transmembrane metalloproteases have been identified on the cell surface of many blood cells. However, the addition of the protease inhibitor aprotinin, leupeptin, pepstatin, phenylmethylsulfonyl fluoride, EDTA, EGTA, or ortho-phenan-

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**Fig 5.** Heparinase treatment of K562 cells reduces binding and crosslinking of 125I-bFGF to FGFRs. (A) K562 cells were incubated in PBS containing 0.1% BSA with or without 5 U/mL heparinase for 2 hours at 25°C to digest cell-surface heparan sulfates. Cells were washed two times with cold PBS to remove cleaved glycosaminoglycan chains. The cells were then incubated with 10 ng/mL 125I-bFGF for 2 hours at 4°C. The cells were washed two times with PBS to remove non-bound ligand, and cell bound radioactivity was measured. The results present the average of duplicate samples. Solid bar, 125I-bFGF alone; open bar, 125I-bFGF in the presence of 100-fold excess of unlabeled bFGF; hatched bar, 125I-bFGF in the presence of 10 μg/mL heparin; stippled bar, 125I-bFGF in the presence of both unlabeled bFGF and heparin. (B) K562 cells were treated with heparinase, chondroitinase, or buffer alone as described in A. The treated cells were incubated with 10 ng/mL 125I-bFGF for 2 hours at 4°C, and the chemical crosslinking agent BS3 was added for 30 minutes at room temperature. The cells were lysed, and lysates were analyzed under reducing conditions on a 3% to 15% gradient SDS-PAGE gel. Each lane contains lysate from 5 × 10^6 cells. Gels were exposed for autoradiography for 2 weeks. Lane a, untreated cells incubated with 125I-bFGF alone; lane b, untreated cells incubated with 125I-bFGF and 1 μg/mL unlabeled bFGF; lane c, untreated cells incubated with 125I-bFGF and 10 μg/mL heparin; lane d, heparinase-treated cells incubated with 125I-bFGF alone; lane e, heparinase-treated cells incubated with 125I-bFGF and 10 μg/mL heparin; lane f, heparinase-treated cells incubated with 125I-bFGF and 1 μg/mL heparin; lane g, heparinase-treated cells incubated with 125I-bFGF and 0.1 μg/mL heparin; lane h, chondroitinase-treated cells incubated with 125I-bFGF alone.
were incubated in medium containing 40 μCi/mL ³²P-O4 for 24 hours to allow cleavage of the receptors. Cells were collected, washed twice with PBS, and incubated with PMA. These results suggest that the decrease in phosphorylation had no effect on the reduction in bFGF receptors after PMA treatment is not the result of proteolytic cleavage of the receptors.

**DISCUSSION**

The leukemia cells examined here, K562, HL60, and DAMI, bind bFGF on three classes of sites: (1) tyrosine kinase signaling receptors, (2) HSPGs, and (3) nonspecific sites. The specific binding to tyrosine kinase receptors and to HSPGs have overlapping properties, making them difficult to separate in direct binding and competition experiments. Both can be inhibited by unlabeled bFGF. Binding to HSPGs is completely inhibited by soluble heparin, and binding to receptors is partially inhibited. Thus, the specific binding measured in the direct binding experiments represents a combination of receptor and HSPG binding. The presence of protein receptors could be verified by crosslinking experiments in which bFGF-receptor complexes with a similar molecular size to those observed in other cell types were detected. The protein receptors seem to account for only a small portion of the total binding. The difference between the results of Fig 5A, where heparinase dramatically decreased specific binding, and Fig 5B, where heparinase treatment reduced the formation of ³²P-aFGF–receptor crosslinked complexes by only 50%, suggests that the majority of the specific binding is due to binding to HSPGs. This conclusion is supported by the Scatchard analysis of binding data, which showed 23 times more low affinity than high affinity binding sites on K562 cells. A low number of protein receptors is also consistent with the finding that although aFGF could be crosslinked to the same protein receptors as bFGF, only low levels of ³²P-aFGF bound to K562 cells in direct binding experiments.

At the time this study began, there were no reports of functional FGFRs in blood cells. However, the two most recently identified members of the FGFR family, FGFR-3 and FGFR-4, have been cloned from K562 cells. It has been reported recently that mRNA for all four FGFRs are expressed in various leukemia cell lines and some peripheral blood cells. Armstrong et al. found high levels of FGFR-3 and FGFR-4 mRNA in erythroid and megakaryocytic leukemia cell lines, suggesting expression of these receptor types may be important for the erythroid and megakaryocytic lineages. Bikfalvi et al. reported that FGFR-1 and FGFR-2 mRNA was present in HEL and DAMI cell lines and in megakaryocytes, platelets, macrophages, granulocytes, T cells, and B cells. These data suggest a possible role for bFGF in the growth or differentiation of other blood cell lineages and the possibility of other receptor types in cells of the megakaryocytic lineage. However, only two studies have demonstrated that the presence of mRNA for FGFRs leads to the presence of functional FGFRs on the cell surface. These studies have shown that K562 cells and HEL cells can bind aFGF. Because FGFR-3 and FGFR-4 are known to have a greater affinity for aFGF than for bFGF, it was not clear from these results that blood-derived cells could interact with bFGF. We have found that the leukemia cell lines K562, DAMI, and HL60 have receptors that interact with bFGF. The recent demonstration that bFGF can inhibit TGF-β-induced differentiation of K562 cells indicates that these receptors can transmit a signal in response to bFGF.

In chemical crosslinking experiments, receptor complexes with molecular weights in the range of 140 to 160 kD were observed in K562 cells. The sizes of these receptor complexes are consistent with those found in other cell types. The range of sizes of these complexes may be due to bFGF binding to different receptor types, as the mRNAs for FGFR-1, FGFR-3, and FGFR-4 have been reported to be present in K562 cells. In addition, the bands may represent binding to alternatively spliced forms of FGFRs, which have been described for FGFR-1 and FGFR-2. Alternative splicing can result in receptor variants possessing either two or three immunoglobulin-like domains in the extracellular region that differ in molecular weight by about 20 kD. The receptor variants of FGFR-1 with two and three immunoglobulin domains both interact with bFGF. The species of FGFR responsible for bFGF binding was not directly addressed in this study. Others have shown that K562 cells express predominately mRNA for FGFR-3 and FGFR-4. These receptors have at least 10-fold lower affinity for bFGF than FGFR-1 and FGFR-2, making their affinity for bFGF closer to the affinity of the bFGF-HSPG interaction. This is
consistent with the fact that binding of bFGF to receptors on K562 cells was partially inhibited by addition of 10 μg/mL heparin, whereas in cells expressing FGFR-1 or FGFR-2, heparin at this concentration does not significantly inhibit binding to receptors (Table 1).

In addition to FGFRs, bFGF-binding HSPGs were found in K562 cells. K562 cells contain greater than 20 times more HSPG binding sites than FGFRs according to Scatchard analysis of 125I-bFGF binding. The presence of cell-surface HSPGs has been previously reported only in blood cells of the B-lymphocyte lineage. As heparin or heparan sulfates seems to be necessary for the long-term biologic activity of bFGF, the presence of HSPGs can have significant effects on the ability of the cells to respond to bFGF. Indeed, removal of the HSPGs by heparinase treatment of the K562 cells reduced their ability to bind bFGF on FGFRs.

Treatment of K562 cells with PMA resulted in a decrease in bFGF binding and loss of ability to form crosslinked bFGF-receptor complexes. We have also observed this effect of PMA in HL60, DAM1, and MO-7E leukemia cell lines, suggesting the effect is not specific for any particular blood cell lineage. In K562 cells, the decreased ability to bind bFGF on receptors occurred rapidly, with 70% loss after 1 hour of treatment. Others have reported that PMA has no effect on FGFR mRNA levels in K562 cells, suggesting that receptors may be affected by phorbol ester treatment at a posttranscriptional step. The bFGF promoter contains a PMA responsive element, and PMA has been shown to increase bFGF mRNA and protein expression in human dermal fibroblasts. Therefore, it is possible that the receptors were downmodulated in an autocrine manner as a result of increased expression of ligand. However, PMA had no effect on the amount of bFGF protein produced by K562 cells, and suramin, which blocks bFGF interaction with receptors, did not prevent FGFR downmodulation in response to PMA. Furthermore, the decrease in receptors was not prevented by treatment with the protein synthesis inhibitor, cyclohexi-

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**Fig 7.** PMA rapidly reduces bFGF binding and crosslinking in K562 cells. (A) K562 cells (2.5 × 10⁶ cells per condition) were incubated at 37°C in medium containing 10⁻⁶ mol/L PMA for the times indicated. The cells were collected, washed twice with PBS, and incubated for 2 hours at 4°C with 10 ng/mL 125I-bFGF in medium containing 0.15% gelatin and 25 mmol/L HEPES, pH 7.0 (solid bars). Some samples contained 1 μg/mL unlabeled bFGF (open bars). Cells were washed twice in PBS, and cell-bound radioactivity was determined. All conditions were performed in duplicate. (B) K562 cells, treated as in A, were incubated with 10 ng/mL 125I-bFGF for 2 hours at 4°C, and the crosslinking agent BS³ was added for 30 minutes at room temperature. The crosslinking reaction was quenched with the addition of 0.1 mol/L glycine, and cells were washed once with PBS and extracted. Cell extracts were analyzed on 3% to 15% gradient SDS-PAGE gels. K562 cells were incubated with 10⁻⁶ mol/L PMA for 0 (lane a), 1 (lane b), 2 (lane c), 6 (lane d), or 12 (lane e) hours. Some cells were incubated with 10⁻⁶ mol/L PMA for 12 hours in the presence of 1 mmol/L suramin (lane f) or 10 μg/mL heparin (lane g). For comparison, K562 cells were treated with 2 ng/mL TGF-β for 2 (lane h) or 6 (lane i) hours. (C) Densitometry of the crosslinked 125I-bFGF-receptor complexes in B (lanes a through e) expressed as a percentage of control lane a. The intensities of the radioactive receptor bands were quantitated by phosphorimager.
mide, demonstrating that new protein synthesis was not involved in the downmodulation. Thus, the downmodulation of FGFR does not appear to be the result of increased synthesis or release of the ligand. In addition, receptors were not decreased in control cells treated with cycloheximide, suggesting that PMA does not act by inhibiting synthesis of FGFR protein.

Activation of PKC in blood-derived cells leads to the loss of cell-surface c-kit receptor, tumor necrosis-α receptor, and colony-stimulating factor-1 receptor through proteolytic cleavage in the extracellular juxtamembrane region of the receptors. Soluble extracellular domains of the receptors are generated that act as binding proteins for the ligands. The loss of bFGF receptors in K562 cells after PMA treatment does not seem to proceed through a similar process. Inhibitors of serine, thiol, and metalloproteases all did not prevent the downmodulation of bFGFR by PMA, and soluble bFGG-binding proteins were not detected in the conditioned medium. FGFRs also may be modulated by phorbol ester through phosphorylation of residues in the cytoplasmic domain of the receptor, as has been described for the epidermal growth factor receptor. The mechanism of downmodulation of FGFRs on K562 cells will be the subject of future investigations.

Recently, a role for FGF in megakaryocytopenesis has been identified, as both sFGF and bFGF can increase human and murine megakaryocyte colony growth. Basic FGF is expressed in both bone marrow stromal cells and megakaryocytes and platelets. These data suggest that bFGF may act in an autocrine or paracrine manner to support megakaryocytic cell growth. Our results demonstrating functional bFGF receptors on DAMI, a megakaryocytic cell line, and K562, a primitive stem cell line capable of differentiating along the megakaryocytic lineage, support a role for bFGF in megakaryocytopenesis.

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