Phospholipase C Release of Basic Fibroblast Growth Factor from Human Bone Marrow Cultures as a Biologically Active Complex with a Phosphatidylinositol-anchored Heparan Sulfate Proteoglycan

Georg Brunner, Janice Gabrilove,* Daniel B. Rifkin, and E. Lynette Wilson

Department of Cell Biology, New York University Medical Center, New York 10016; and * Memorial Sloan-Kettering Cancer Center, New York 10021

Abstract. Basic fibroblast growth factor (bFGF) is a potent mitogen for human bone marrow stromal cells and stimulates haematopoiesis in vitro. We report here that primary human bone marrow cultures contain bFGF and express heparin-like bFGF binding sites on the cell surface and in the extracellular matrix (ECM). bFGF bound predominantly to a 200-kD cell surface heparan sulfate proteoglycan (HSPG), which was also found in conditioned medium.

bFGF was released from bone marrow cultures by incubation with phosphatidylinositol-specific phospholipase C (PI-PLC) and, less efficiently, by plasmin. Solubilized bFGF was found as a complex with the 200-kD HSPG. The complex was biologically active as shown by its ability to stimulate plasminogen activator production in bovine aortic endothelial cells. bFGF-HSPG complexes of bovine endothelial cells, however, were not released by PI-PLC.

While only trace amounts of the bFGF-binding 200-kD HSPG were released spontaneously from bone marrow cultures, incubation with PI-PLC solubilized almost all of the 200-kD HSPG. The HSPG could be metabolically labeled with ethanolamine or palmitate, which was partially removed by treatment with PI-PLC. These findings indicate linkage of the HSPG to the cell surface via a phosphatidylinositol anchor. Plasmin released the 200-kD HSPG less efficiently than PI-PLC.

We conclude that HSPGs of human bone marrow serve as a reservoir for bFGF, from which it can be released in a biologically active form via a dual mechanism; one involving a putative endogenous phospholipase, the other involving the proteolytic cascade of plasminogen activation.

PROLIFERATION and differentiation of haematopoietic stem cells in bone marrow is dependent on the continuous supply of growth factors such as the colony stimulating factors (Metcalf, 1986; Clark and Kamen, 1987), and close association between the primitive progenitor cells and bone marrow stromal cells is required (Dexter et al., 1977; Dexter, 1982). It is thought that stromal cells produce growth factors essential for haematopoietic cell development and that these are deposited in the extracellular matrix (ECM)[1]. The fact that granulocyte-macrophage colony stimulating factor and interleukin-3 have been shown to bind in a biologically active form to heparan sulfate in bone marrow stroma (Gordon et al., 1987; Roberts et al., 1988) supports this hypothesis.

Basic fibroblast growth factor (bFGF), a ubiquitous multifunctional growth factor, is found in many tissues, tumors, and cell lines (for a review see Rifkin and Moscatelli, 1989). In endothelial cells in vitro, bFGF acts as an autocrine growth factor (Sato and Rifkin, 1988) and induces cell growth, cell migration, DNA synthesis, plasminogen activator (PA), and metalloproteinase production, indicating the involvement of this growth factor in neovascularization and wound repair (Rifkin and Moscatelli, 1989). In human bone marrow cultures, bFGF stimulates cell growth and the formation of a primary stromal cell layer and delays senescence of stromal cultures (Oliver et al., 1990). It also enhances myelopoiesis when added to long-term human bone marrow cultures in vitro (Wilson et al., 1991). Whereas bFGF alone had no effect on colony formation by progenitor cells, it enhanced synergistically the frequency of colonies produced by other haematopoietic growth factors (Gabbianelli et al., 1990; Wilson et al., 1991).

bFGF is not found in significant amounts in serum or cell culture supernatants (Gauthier et al., 1987) but is deposited into the ECM of endothelial cells (Vlodavsky et al., 1987).

1. *Abbreviations used in this paper*: BAE, bovine aortic endothelial; BCE, bovine capillary endothelial; bFGF, basic fibroblast growth factor; ECM, extracellular matrix; HSPG, heparan sulfate proteoglycan; KIU, Kallikrein inhibitor unit; PA, plasminogen activator; PI, phosphatidylinositol; PLC, phospholipase C.

ECM-bound bFGF provides a reservoir of biologically active growth factor, which is able to mediate long-term biological effects (Flaumenhaft et al., 1989). Nonreceptor bFGF binding sites, heparan sulfate proteoglycans (HSPGs), are present on the cell surface as well as in the ECM. The growth factor is released from these sites as a biologically active growth factor-heparan sulfate proteoglycan (HSPG) complex by the serine proteinase plasmin (Saksela and Rifkin, 1990) and by heparanases (Ishai-Michaeli et al., 1990). HSPGs are also modulators of the biological activity of bFGF as heparin and heparan sulfate protect the growth factor from proteolytic inactivation (Saksela et al., 1988). Polyanionic glycosaminoglycans also increase the diffusion of bFGF within an endothelial cell monolayer, thereby extending the range of biological action of this factor (Flaumenhaft et al., 1990). In addition, heparin-like molecules were reported to be accessory molecules necessary for bFGF binding to its high-affinity receptor (Yayon et al., 1991). This indicates that bFGF complexes with HSPGs or glycosaminoglycans serve as the biologically active forms of this growth factor.

In this paper, we demonstrate that bFGF binds to HSPGs of human primary bone marrow cultures. The major bFGF-binding HSPG, which has a molecular size of ~200 kD, is linked to the stromal cell surface via a phosphatidylinositol (PI) anchor. Consequently, bFGF is released as biologically active growth factor-HSPG complex not only by plasmin, but more efficiently by PI-specific phospholipase C (PI-PLC). These results suggest a dual mechanism of bFGF release in human bone marrow cultures. The first and more efficient mechanism involves the release of PI-linked bFGF-HSPG complexes from the stromal cell surface by a putative endogenous phospholipase. The second mechanism involves the proteolytic cascade of plasminogen activation and plasmin-mediated ECM degradation also leading to the solubilization of bFGF-HSPG complexes.

Materials and Methods

Reagents

Recombinant bFGF was a generous gift of Synergen Inc. (Boulder, CO) (Sommer et al., 1987). [^{125}I]bFGF (7.9 mCi/mg) was kindly provided by Dr. D. Moscatelli (NYU Medical Center, New York). Sodium [^{35}S]sulfate (250–1,000 mCi/mmol) was obtained from Du Pont (Wilmington, DE), [1-^{3}H]ethanolamine (30.4 Ci/mmol) and [9,10(n)-^{3}H]palmitic acid (53.6 Ci/mmol) from Amersham (Arlington Heights, MA). PI-PLC (600 u/mg; protease-free) and phosphatidylcholine PLC (PC-PLC; 2,000 μ/mg) were purchased from Boehringer (Mannheim, FRG). Plasminogen was purified from outdated human plasma (Deutsch and Mertz, 1970), and urokinase-type PA was purchased from Leo Pharmaceuticals (Denmark). Affinity-purified polyclonal antibodies against recombinant bFGF (Dennis and Rifkin, 1990) were a generous gift of P. A. Dennis (NYU Medical Center, New York). Goat anti-rabbit IgG coupled to alkaline phosphatase was obtained from Promega Biotec (Madison, WI). Plasmin (4 μ/mg), its chromogenic substrate D-Val-Leu-Lys p-nitroanilide, heparinase (12,000 μ/mg), aprotinin (9,900 KIU/mg), fatty-acid free BSA, 4-methylumberylliferyl β-D-xyloside, hydrocortisone, heparin, 5-bromo-4-chloro-3-indolyl phosphate, nitro blue tetrazolium, and protein A–Sepharose CL-4B were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell Culture

Human bone marrow cells were obtained from healthy adult volunteers following informed consent. Buffy coat cells were seeded in alpha modified Eagle's medium (Flow Laboratories, McClean, VA) containing 12.5% FCS (Intergen Company, New York, NY), 12.5% horse serum (Gibco Laboratories, Grand Island, NY), 10^{-6} M hydrocortisone, 10^{-4} M 2-mercaptoethanol, 2 mM L-glutamine, and antibiotics. After 3–4 wk an adherent stromal cell layer containing haematopoietic progenitor cells was present (Dexter, 1982). All experiments were performed using confluent cell layers of the same frozen stock of primary bone marrow cells. For studies on bFGF binding or on the enzymatic release of bFGF or HSPG into the medium, the cultures were washed with PBS and incubated in the medium specified above but without serum. Cell lysates were prepared by incubating the cell layer with PBS containing 0.5% Triton X-100 for 15 min at 37°C. The residual stromal cell matrix, still containing fragments of the lysed cells, was extracted for 15 min at 37°C with reducing SDS-PAGE sample buffer. This extract represented a mixture of cellular and matrix constituents because of the incomplete separation of these components.

Bovine aortic (BAE) and capillary endothelial (BCE) cells were isolated as described previously (Folkman et al., 1979) and grown at 37°C in alpha modified Eagle's medium supplemented with 5% calf serum, 2 mM L-glutamine, and antibiotics. Cells were used for experiments between passage 5 and 10.

Metabolic Labeling of Bone Marrow Cells

For the labeling of the glycosaminoglycan side chains of proteoglycans, cells were grown until confluent in 24-well Linbro plates and incubated overnight with [^{35}S]sulfate (40 μCi/ml) in serum-free bone marrow culture medium (250 μl/well).

For the labeling of the PI anchor of the 200-kD HSPG, 5 mCi of [^{3}H]palmitic acid were dried under nitrogen and dissolved in 100 μl of cold 95% ethanol. A palmitate-BSA complex was prepared by adding 1.25 ml of a solution of 20 mg/ml of fatty-acid free BSA in 10 mM sodium phosphate buffer, pH 7.0, containing 150 mM sodium chloride to the [^{3}H]palmitic acid. The complex was allowed to form for 1 h at room temperature. Cells were grown until confluent in an 80-cm^2 flask and were incubated overnight with the palmitate-BSA complex (0.8 mCi/ml) in serum-free bone marrow culture medium (6.25 ml/flask) containing 200 KIU/ml aprotinin. Alternatively, cells were incubated overnight with [^{3}H]ethanolamine (167 μCi/ml) in serum-free medium containing 200 KIU/ml of aprotinin and 20 ng/ml of bFGF to achieve maximal incorporation of the label into the HSPG. Cell lysates were prepared as described above and PMSF was added to a final concentration of 1 mM.

[^{125}I]bFGF Binding Assay

The [^{125}I]bFGF binding assay was a modification of the procedure described by Moscatelli and Devesly (1990). Bone marrow cells were cultured in 96-well plates until confluent and incubated with 0–1,000 ng/ml of bFGF containing 7.9–79 nCi/ml [^{125}I]bFGF (7.9 mCi/mg) in serum-free bone marrow cell culture medium (100 μl/well) supplemented with 0.15% gelatin and 25 mM Hepes buffer, pH 7.2. After incubation on a shaker for 2 h at 4°C, excess bFGF was removed by extensive washing of the cell layers with PBS followed by determination of the amount of radioactivity in the cell extract and the ECM, respectively, using a Packard MULTI-PRIAS 1 gamma counter.

Immunoprecipitation of bFGF-HSPG Complexes

HSPGs were metabolically labeled overnight with [^{35}S]sulfate, [^{3}H]palmitic acid, or [^{3}H]ethanolamine as described above. The labeled HSPGs were precipitated by addition of bFGF-anti-bFGF IgG complexes immobilized on protein A–Sepharose beads (Saksela and Rifkin, 1990). The precipitates were analyzed by SDS-PAGE (Laemmli, 1970) in 3-16% acrylamide gradient gels (Saksela and Rifkin, 1990).

bFGF Ligand Binding Assay

Serum-free culture supernatants, cell lysates, and ECM extracts of primary bone marrow cell cultures were subjected to SDS-PAGE in 3–16% acrylamide gradient gels. Proteins were transferred onto nitrocellulose filters (Schleicher & Schuell, Keene, NH) according to Towbin et al. (1979) at 250–500 mA for 12–36 h. Unoccupied protein binding sites on the nitrocellulose filters were blocked with 5% dry milk in PBS for 1 h. After incubation with bFGF (1 μg/ml in PBS containing 5% dry milk) for 1 h, bound bFGF was detected by incubation with affinity-purified rabbit anti-bFGF IgG (5 μg/ml in PBS containing 5% dry milk) and goat anti-rabbit IgG coupled to alkaline phosphatase (1:10,000 in PBS containing 5% dry milk).

Alkaline phosphatase activity was visualized using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium. All incubations were performed at room temperature.

bFGF Immunoblotting

Immunoblotting was performed essentially as described for the bFGF ligand binding assay (see above) except that the incubation step with bFGF was omitted.

PA Assay

PA was assayed via the activation of plasminogen to plasmin followed by the cleavage of a chromogenic plasmin substrate as described previously (Brunner et al., 1989). BAE cells were cultured in 96-well plates until confluent and incubated overnight with bFGF-containing samples. Cells were then lysed by addition of 30 mM Tris-HCl buffer, pH 7.5, containing 60 mM sodium chloride and 0.5% Triton X-100 (60 μl/well). After incubation for 15 min at 37°C, PA activity in the cell lysates was determined directly in the wells in a coupled chromogenic assay by adding 20 μl of human plasminogen (500 μg/ml) and 20 μl of D-Val-Leu-Lys p-nitroanilide (3.5 mM) as substrates. After incubation for 30 min at 37°C, optical density at 405 nm was determined using a microtiter plate reader. The values were corrected for plasmin contamination of the reagents (buffer control) and for plasmin-independent turnover of the chromogenic substrate (controls lacking plasminogen).

Results

Demonstration of Endogenous bFGF in Bone Marrow Cultures

It is known that bFGF is a potent mitogen for primary human bone marrow cells (Oliver et al., 1990) and that this growth factor promotes myelopoiesis in long-term human bone marrow cultures (Wilson et al., 1991). To investigate whether bFGF might be produced as an autocrine growth factor by bone marrow cultures themselves, we examined Triton X-100 cell lysates and ECM extracts for the presence of bFGF immunoreactivity. In immunoblotting experiments bFGF was found in ECM extracts at a molecular weight position of 18 kD (Fig. 1, lane *2*), but was undetectable in cell lysates (Fig. 1, lane *1*). A less intense band was also observed at ~32 kD. This may represent a dimer of bFGF (our unpublished data). Since bFGF is not present in serum, this result suggested that bFGF was produced by the bone marrow cultures.

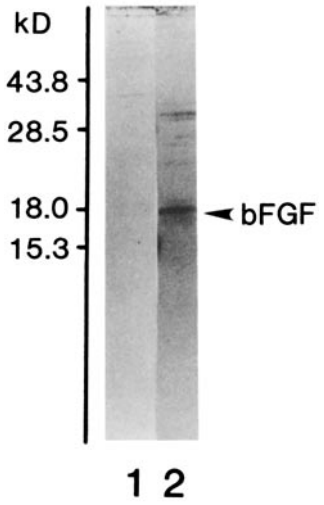

Figure 1. Demonstration of bFGF in primary human bone marrow cultures. Cells were cultured until confluent and lysed for 15 min at 37°C in PBS containing 0.5% Triton X-100 (5 × 10^6 cells/ml). The residual stromal cell matrix was extracted for 15 min at 37°C with a corresponding volume of reducing SDS-PAGE sample buffer. Aliquots of cell lysates (lane *1*) and ECM extracts (lane *2*) (each corresponding to 4.5 × 10^5 cells) were analyzed by SDS-PAGE in 15% gels under reducing conditions. bFGF was detected by immunoblotting using affinity-purified anti-bFGF IgG (5 μg/ml) and anti-rabbit IgG coupled to alkaline phosphatase (1:10,000). Normal rabbit IgG (5 μg/ml) was used as a control and no bands were evident (not shown).

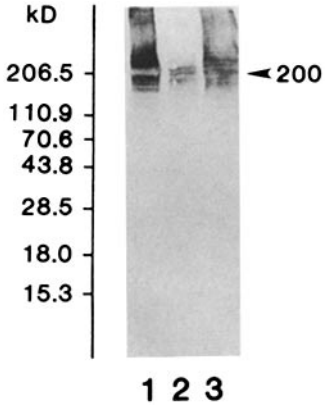

Figure 2. Identification of bFGF-binding HSPGs in bone marrow cultures. Cells were cultured in serum-containing medium in 24-well plates until confluent and extracted (250 μl/well) as described in Fig. 1. Aliquots (90 μl) of cell extracts, ECM, or 24-h serum-free conditioned medium (250 μl/well) were analyzed by SDS-PAGE in 3–16% gel gradients under reducing conditions. bFGF-binding molecules were detected by ligand blotting using recombinant bFGF (1 μg/ml), affinity-purified anti-bFGF IgG (5 μg/ml), and anti-rabbit IgG coupled to alkaline phosphatase (1:10,000). No bands were seen using normal rabbit IgG as a control, or if the incubation step with bFGF was omitted (not shown). (lane *1*) Conditioned medium; (lane *2*) cell extract; (lane *3*) ECM extract.

Identification of Heparin-like bFGF Binding Sites in Bone Marrow Cultures

To demonstrate the presence of bFGF binding sites in primary human bone marrow cultures, exogenous [^{125}I]bFGF was added to confluent cell layers and cell-bound bFGF was quantified in Triton X-100 cell lysates. The residual ECM was then extracted with reducing SDS-PAGE sample buffer. This extract represented a mixture of matrix and cellular components, since the detergent treatment did not result in complete removal of cells sequestered in the ECM.

Both compartments, bone marrow cells and the ECM, contained bFGF binding sites, which were not saturable up to a bFGF concentration of 1 μg/ml (results not shown). After addition of bFGF, ~13% of the growth factor was recovered in the cell lysate and 27% in the ECM extract (i.e., a total of 40%). The bFGF binding capacity of bone marrow cultures calculated from these values exceeded 70 ng/10^5 cells.

bFGF has been shown to bind to heparan sulfate and HSPGs of endothelial cells (Saksela et al., 1988; Saksela and Rifkin, 1990), and bFGF binding to these sites can be competed by heparin (Flaumenhaft et al., 1989). We therefore examined our bone marrow cultures for evidence of heparin-like bFGF binding sites, and noted that in the presence of 100 μg/ml of heparin, bFGF binding was almost completely abrogated (results not shown). More than 95% of the cellular and 98% of the matrix binding sites were competable by heparin. This is consistent with previous reports on bFGF binding to cultures of BCE cells (Moscatelli, 1987, 1988).

To characterize further the heparin-like bFGF binding sites in primary bone marrow cultures, we prepared serum-free conditioned medium, Triton X-100 cell lysates, and an extract of the residual ECM. These fractions were screened for bFGF-binding molecules by SDS-PAGE followed by ligand blotting (see Materials and Methods).

Using this technique, bFGF was shown to bind to a series of molecules of a molecular size of ~200 kD in both the cell

extract and ECM (Fig. 2, lanes *2* and *3*) representing differentially glycosylated variants of a proteoglycan (see below). In addition, a small amount of binding to species of higher molecular weight was observed in the ECM sample and in the medium (Fig. 2, lanes *1* and *3*). The 200-kD molecule was spontaneously released from the cultures, since it was also present in conditioned medium (Fig. 2, lane *1*).

The 200-kD bFGF-binding molecule was identified as a HSPG, as it was metabolically labeled with [^{35}S]sulfate (see Figs. 5 and 8), and bFGF binding was abolished by pretreatment of the cultures with heparinase (results not shown). Furthermore, growth of the cells in the presence of 4-methylumberylliferyl β-D-xyloside, which inhibits the attachment of the glycosaminoglycan side chains to the core protein of proteoglycans (Schor and Schor, 1988), almost completely abrogated bFGF binding to the 200-kD molecule in ligand blotting experiments (not shown). Finally, the molecule was sensitive to proteolytic digestion, as it was released from the cultures by plasmin treatment (see Fig. 7). The 200-kD bFGF-binding HSPG of bone marrow cultures was similar in size to that found in BCE cell cultures (250 kD) (Saksela and Rifkin, 1990).

Enzymatic Release of bFGF from Bone Marrow Cultures

To have a biological effect in bone marrow cultures, bFGF has to be released from its heparin-like binding sites. Since the HSPG described above constitutes the major binding site for bFGF in bone marrow cultures (Fig. 2 and data not shown), the solubilization of these HSPGs might represent a mechanism for the release of bFGF from this reservoir. It has been shown that bFGF bound to HSPGs of the endothelial cell matrix is solubilized by plasmin (Saksela and Rifkin, 1990) or heparanases (Ishai-Michaeli et al., 1990). HSPGs in liver, brain, ovaries, and lung, however, have been proposed to be linked to the cell surface via a PI anchor (Ishihara et al., 1987; Carey and Evans, 1989; Yanagashita and McQuillan, 1989; David et al., 1991). We therefore investigated whether bFGF bound to HSPGs can be released from bone marrow cultures by treatment with various enzymes.

bFGF (1 μg/ml) was added to primary bone marrow cultures for 1 h at 37°C. After extensive washing the cultures were subsequently incubated for a further 2 h with heparinase (0.5 μg/ml), plasmin (1 μg/ml), or PI-PLC (0.5 μg/ml). bFGF release by these enzymes was demonstrated by SDS-PAGE and immunoblotting of serum-free conditioned media and the corresponding Triton X-100 cell lysates. After incubation for 2 h with plasmin or heparinase, bFGF release into the medium was slightly increased compared to control cultures (results not shown). In contrast, incubation of the cultures with PI-PLC considerably increased the amount of bFGF found in the medium as compared to the spontaneous release of bFGF (Fig. 3, lane *6* compared to lane *5*). The release of bFGF into the medium was associated with its corresponding disappearance from the cell extracts (Fig. 3, lane *3* compared to lane *2*).

To examine the time course of the release of bFGF by PI-PLC, we incubated primary bone marrow cultures with [^{125}I]bFGF (100 ng/well; 26,400 cpm/well) as described above. After extensive washing, the cultures were subsequently incubated with PI-PLC (0.5 μg/ml) and the amount of labeled bFGF in conditioned media, cell extracts, and residual ECM was determined at different time intervals. Approximately 66% (corresponding to 4.4 ng/well) of the cell-bound bFGF was released by PI-PLC after incubation for 2 h (Fig. 4). Some of the residual bFGF contained in the cell lysates might represent bFGF, which was internalized by the cells during the incubation period. An initial small decrease in the amount of bFGF bound to the ECM was observed, which could result from the incomplete separation of cells and matrix as mentioned previously.

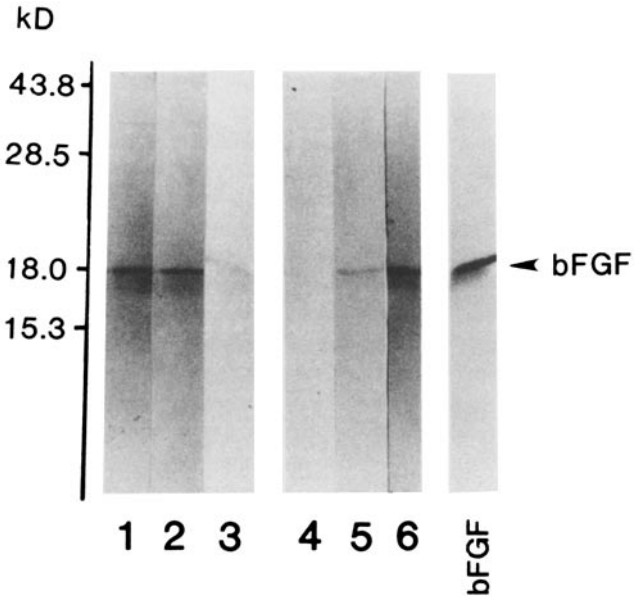

Figure 3. Enzymatic release of bFGF from bone marrow cultures. Cells were cultured in serum-containing medium in 24-well plates until confluent and 1 μg/ml of recombinant bFGF in serum-free medium containing 0.15% gelatin (250 μl/well) was added for 1 h at 37°C. After extensive washing with PBS, cells were either rinsed briefly with serum-free medium (250 μl/well) without a further incubation (lanes *1* and *4*), or incubated for a further 2 h with medium alone (lanes *2* and *5*) or 0.5 μg/ml of PI-PLC (lanes *3* and *6*). All incubations were performed in the presence of 100 KIU/ml of aprotinin. Aliquots (90 μl) of conditioned media (lanes *4–6*) or of the corresponding cell lysates (lanes *1–3*), prepared as described in Fig. 1 (250 μl/well), were analyzed by SDS-PAGE in a 15% gel followed by immunoblotting as described in Fig. 1. The last lane contained 50 ng of recombinant bFGF.

bFGF Is Released as a Complex with the 200-kD HSPG by PI-PLC and Plasmin

The highly efficient release of bFGF by PI-PLC suggested that the major bFGF binding site, the 200-kD HSPG, might be linked to the cell surface via a PI anchor. If this was the case, bFGF would be solubilized by PI-PLC as a complex with the HSPG. To test this hypothesis, we attempted to isolate bFGF-HSPG complexes from the supernatants of enzymatically treated cultures.

HSPGs of primary bone marrow cultures were metabolically labeled overnight with [^{35}S]sulfate and incubated with 1 μg/ml of [^{125}I]bFGF for 1 h. After extensive washing with PBS, the cultures were then incubated with PI-PLC, plasmin, or heparinase to release double-labeled bFGF-HSPG complexes. The complexes were precipitated from the supernatants by addition of protein A–Sepharose beads coated

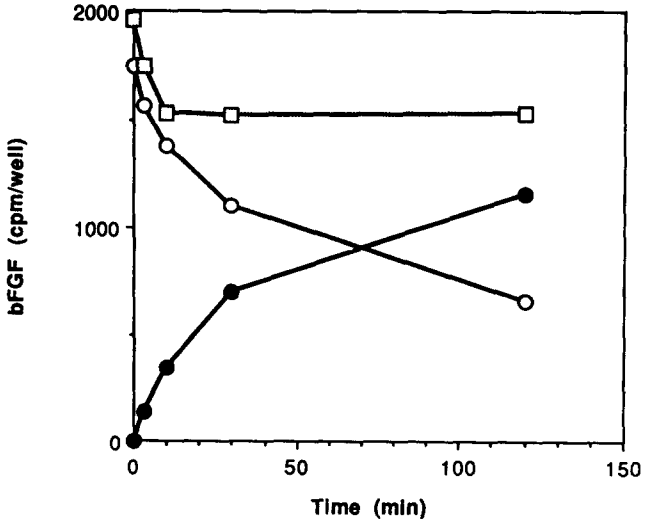

Figure 4. Time course of bFGF release from bone marrow cultures by PI-PLC. Cells were cultured in serum-containing medium in 96-well plates until confluent and cultures were incubated with [^{125}I]bFGF (1 μg/ml in serum-free medium containing 0.15% gelatin; 264,000 cpm/ml; 100 μl/well) for 1 h at 37°C. After extensive washing with PBS, cultures were incubated with 0.5 μg/ml of PI-PLC (100 μl/well) containing 100 KIU/ml of aprotinin and extracted (100 μl/well) as described in Fig. 1. Radioactivity in conditioned medium (●), cell extract (○), or ECM (□) was determined by counting the samples in a gamma counter. Values represent the mean value of duplicate samples, and similar results were obtained in two separate experiments.

with anti-bFGF IgG and analyzed by SDS-PAGE under reducing conditions. This procedure separated the complexes into two components – the 200-kD [^{35}S]HSPG and the 18-kD [^{125}I]bFGF. Supernatants of cultures incubated with PI-PLC contained considerably higher amounts of bFGF-HSPG complexes than the medium control (Fig. 5 *A*, lane *5* compared to lane *2*). This is in agreement with the increased release of bFGF by PI-PLC shown in Fig. 3. Whereas plasmin also released an increased amount of bFGF-HSPG complexes compared to control cultures (Fig. 5, lane *4* compared to lane *2*), heparinase did not significantly alter the amount of bFGF released spontaneously from the cultures (Fig. 5, lane *3* compared to lane *2*). The presence of both bFGF and HSPG in the precipitates indicated the release of intact bFGF-HSPG complexes from bone marrow cultures by treatment with PI-PLC and, to a lesser extent, by plasmin. The amount of bFGF released under the different conditions used was quantitated by densitometric scanning of the bFGF band (Fig. 5 *C*). The results indicated that PI-PLC and plasmin were more effective in releasing bFGF than heparinase.

Using the same technique, we isolated bFGF-HSPG complexes released from BAE and BCE cell cultures by PI-PLC treatment. The bFGF-binding HSPGs of these cultures differed somewhat in their molecular size from those of bone marrow cultures (Fig. 5 *B* and Saksela and Rifkin, 1990). It appeared, however, that the spontaneous release of bFGF was not significantly increased by incubation with PI-PLC (Fig. 5, lanes *2* and *4* compared to control lanes *1* and *3*). This conclusion was also supported by the densitometric scanning of the bFGF band (Fig. 5 *C*). A band of increased intensity was observed at the top of the gel after addition of PI-PLC to BAE cells (Fig. 5 *B*, lane *2*). This was not reproducible and most likely comprised aggregates of proteoglycans which were unable to enter the gel.

These results suggest the existence of different types of bFGF-binding HSPGs in primary human bone marrow cultures and in bovine endothelial cell lines which differ in their sensitivity towards release by PI-PLC.

The Released bFGF-HSPG Complex Is Biologically Active

bFGF is known to stimulate PA production in endothelial cells (Moscatelli et al., 1986; Presta et al., 1986) and, as mentioned previously, bFGF complexed to HSPGs is most likely the biologically active form of this growth factor. To determine whether the bFGF, which is released as a complex with the 200-kD HSPG (see Fig. 5 *A*), is biologically active, we incubated BAE cells overnight with bFGF-HSPG complexes that had been released from bone marrow cultures after incubation with PI-PLC. The cells were then lysed in Triton X-100, and the PA activity in the lysates was determined using plasminogen and D-Val-Leu-Lys p-nitroanilide as substrates.

bFGF (1 and 3 ng/ml) increased PA production by BAE cells 2–2.3-fold (Fig. 6, columns *2* and *3* compared to column *1*). bFGF-HSPG complexes, released from bone marrow cultures by treatment with PI-PLC, stimulated PA production 3.3-fold at a dilution of 1:250 (Fig. 6, column *8* compared to column *1*) and 2.4-fold at 1:1,250 (Fig. 6, column *9* compared to column *1*). At these dilutions, control conditioned medium as well as PI-PLC itself (0.5 μg/ml) did not affect PA activity (Fig. 6, columns *4* and *5* compared to column *1*, and results not shown). These results indicate that the amount of bFGF released by PI-PLC exceeded 1 μg/ml and was significantly higher than that released spontaneously from the cultures. To exclude the possibility that the stimulation of PA production was caused by other compounds released from the bone marrow cells, we attempted to reverse the stimulatory effect by inhibitory antibodies to bFGF. The basal PA activity of BAE cells was almost completely suppressed by anti-bFGF antiserum (Fig. 6, columns *6* and *7* compared to *4* and *5*). Apparently the basal PA activity observed was due to the endogenous release of bovine bFGF-HSPG complexes (see Fig. 5 *B*). Similarly, the PA activity of BAE cells stimulated by the PI-PLC releasates from the bone marrow cultures was almost completely suppressed by anti-bFGF antiserum (Fig. 6, columns *10* and *11* compared to columns *8* and *9*). These results indicated the presence of active bFGF in the conditioned media. PA induction due to the release of endogenous bovine bFGF-HSPG complexes catalyzed by the PI-PLC contained in the stromal cell conditioned media can be excluded, since a concentration of PI-PLC 1,000-fold higher than used in these experiments did not release detectable amounts of bFGF from endothelial cells after an incubation period of 2 h (Fig. 5 *B*).

The 200-kD HSPG Is Linked to the Cell Surface via a PI Anchor

The release of the bFGF-HSPG complexes from primary bone marrow cultures by PI-PLC (see Fig. 5 *A*), but not by phosphatidylcholine-specific PLC (results not shown), indicated the presence of a PI-containing cell surface anchor for the 200-kD bFGF-binding HSPG. One would therefore ex-

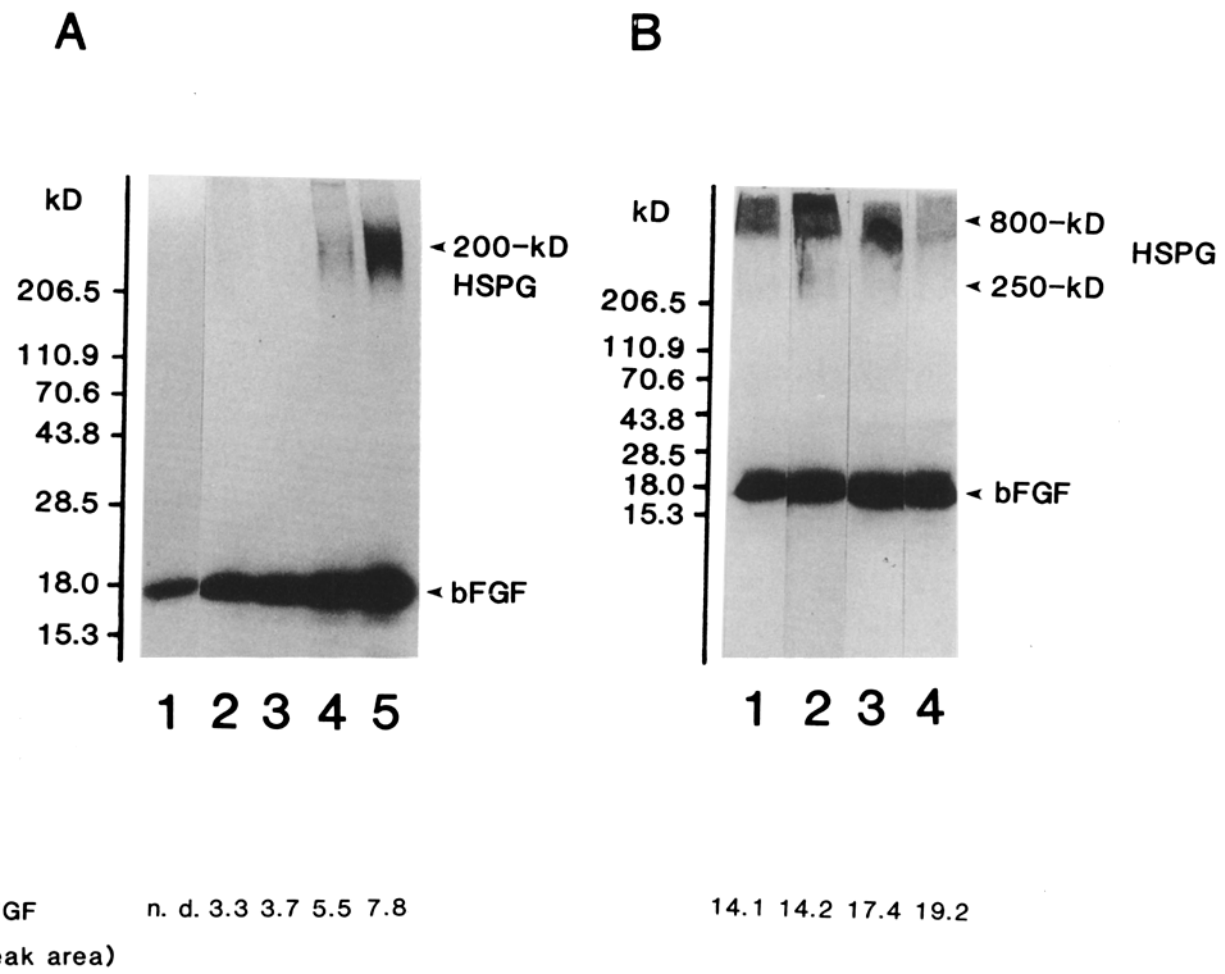

Figure 5. Demonstration of enzymatically released bFGF-HSPG complexes. Cells were cultured in serum-containing medium in 24-well plates until confluent and proteoglycans were metabolically labeled overnight with [^{35}S]sulfate (40 μCi/ml in serum-free medium; 250 μl/well). [^{125}I]bFGF (1 μg/ml in serum-free medium containing 0.15% gelatin; 100,000 cpm/ml; 250 μl/well) was then added to the cultures for 1 h at 37°C. (*A*) Bone marrow cultures. After extensive washing with PBS, double-labeled bFGF-HSPG complexes were released by incubation for 2 h at 37°C with medium alone (250 μl/well) (lane *2*), 0.5 μg/ml of heparinase (lane *3*), 1 μg/ml of plasmin (lane *4*), or 0.5 μg/ml of PI-PLC (lane *5*). All incubations, except for those containing plasmin, were performed in the presence of 100 KIU/ml of aprotinin. The complexes were then isolated by precipitation with protein A-Sepharose beads coated with anti-bFGF IgG, and analyzed by SDS-PAGE in 3–16% gel gradients followed by autoradiography. Lane 1 contained 0.2 ng (3,500 cpm) of [^{125}I]bFGF. No labeled proteins were precipitated using protein A-Sepharose beads coated with normal rabbit IgG (not shown). (*B*) Endothelial cell cultures. Incubation of BAE cells with medium alone (lane *1*) or PI-PLC (lane *2*). Incubation of BCE cells with medium alone (lane *3*) or PI-PLC (lane *4*). (*C*) The intensity of the [^{125}I]bFGF band was determined by scanning of the autoradiograph. The peak area of the scans was expressed in relative units and is shown below the corresponding lanes.

pect that the HSPG itself should be released by PI-PLC. Moreover, the HSPG should be metabolically labeled with PI anchor components, such as ethanolamine and fatty acids.

To test the first assumption, primary bone marrow cultures were incubated at 37°C with medium, plasmin, or PI-PLC, and after various time intervals the resulting conditioned medium as well as the corresponding cell extracts were analyzed by bFGF ligand blotting. All incubations, except for those containing plasmin, were performed in the presence of 100 KIU/ml of aprotinin. The bone marrow cultures spontaneously released trace amounts of the 200-kD bFGF-binding HSPG into the medium (Fig. 7 *A*, lanes *1–4*). Incubation of the cell layer with 1 μg/ml of plasmin for 2 h resulted in a significant increase in the release of the 200-kD HSPG (Fig. 7, lane *8* compared to *4*). In contrast, PI-PLC (0.5 μg/ml) solubilized considerable amounts of the 200-kD HSPG after 3–10 min (Fig. 7, lanes *9* and *10* compared to control lanes *1* and *2* and plasmin lanes *5* and *6*), and the release was almost complete after 2 h (Fig. 7, lane *12*). The release of the 200-kD HSPG into the medium was associated with a marked reduction in the corresponding cell-associated HSPG (Fig. 7 *B*).

To determine the percentage of the total proteoglycan that was released by PI-PLC, we labeled primary bone marrow cultures overnight with [^{35}S]sulfate. Cultures were then incubated with PI-PLC and the radioactivity in the supernatant, cell extracts and residual ECM was determined at different time intervals. After a 2 h incubation with PI-PLC, a period which permitted almost complete release of the 200-kD bFGF-binding HSPG (see Fig. 7, lane *12*), ~40%

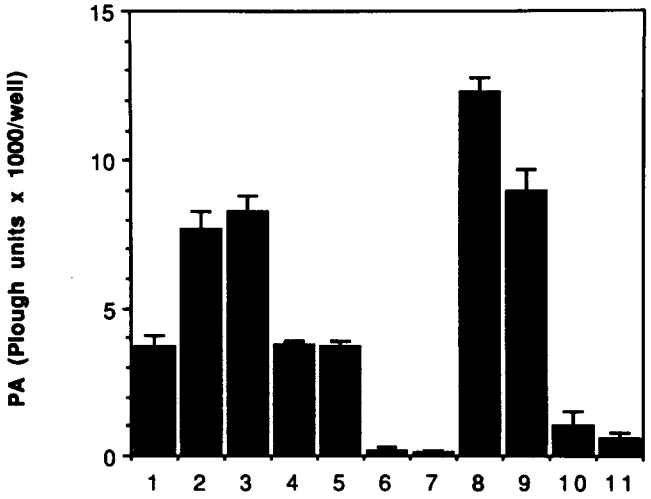

Figure 6. PA induction in BAE cells by bone marrow-derived bFGF-HSPG complexes. Bone marrow cultures were preincubated with 10 μg/ml of bFGF in serum-free BAE medium containing 0.15% gelatin, and bFGF-HSPG complexes were released by incubation with medium alone (columns *4–7*) or PI-PLC (columns *8–11*) as described in Fig. 5. Aliquots (100 μl) of serum-free control medium (columns *1–3*) or of diluted bone marrow culture conditioned media (columns *4–11*) were added to confluent BAE monolayers seeded in 96-well plates and incubated overnight at 37°C. PA activity in BAE cell lysates was then assayed for 30 min at 37°C as described in Materials and Methods and expressed as the mean value of triplicate wells ± SEM. Control medium (column *1*); medium containing 1 (column *2*) or 3 ng/ml (column *3*) of recombinant bFGF; bone marrow culture conditioned media diluted 1:250 (columns *4* and *6*) or 1:1,250 (columns *5* and *7*); PI-PLC-conditioned media diluted 1:250 (columns *8* and *10*) or 1:1,250 (columns *9* and *11*). Conditioned media contained either anti-bFGF antiserum 1:50 (columns *6, 7, 10, 11*) or preimmune rabbit serum 1:50 (columns *4, 5, 8, 9*).

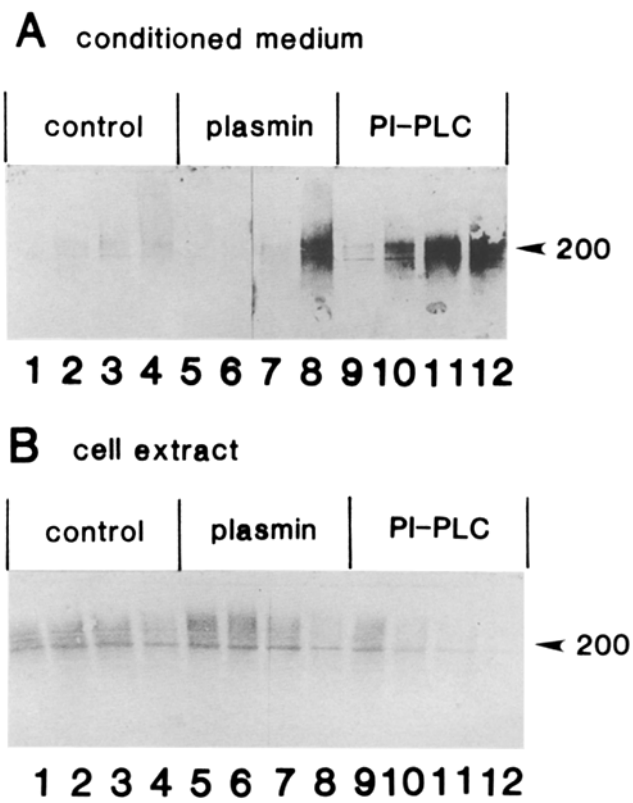

Figure 7. Time course of HSPG release from bone marrow cultures by PI-PLC or plasmin. Experimental conditions were as described in Fig. 3. The medium control and the PI-PLC incubation mixture contained 100 KIU/ml of aprotinin. Cells were incubated with medium alone (lanes *1–4*), plasmin (lanes *5–8*), or PI-PLC (lanes *9–12*) for 3 min (lanes *1, 5, 9*), 10 min (lanes *2, 6, 10*), 30 min (lanes *3, 7, 11*), or 120 min (lanes *4, 8, 12*). Serum-free conditioned media (*A*) and the corresponding cell lysates (*B*) were analyzed by bFGF ligand blotting as described in Fig. 2.

of the total cellular [^{35}S]sulfate label was solubilized. This appeared to be derived predominantly from the cells, as there was little change in the [^{35}S]sulfate content of the matrix (results not shown).

To test the second assumption, namely that the 200-kD HSPG is linked to the cell surface via a PI anchor, primary bone marrow cells were metabolically labeled overnight with 167 μCi/ml of [^{3}H]ethanolamine, with 0.8 mCi/ml of a [^{3}H]palmitic acid-BSA complex, or with 40 μCi/ml of [^{35}S]sulfate. bFGF-binding molecules were isolated from cell lysates by the addition of protein A-Sepharose coated with bFGF-anti-bFGF IgG complexes. The precipitates were analyzed by SDS-PAGE followed by autoradiography. The 200-kD HSPG labeled by ethanolamine (Fig. 8, lane *2*), by palmitic acid (lane *2*), or by sulfate (lane *3*) was precipitated by this technique. Incubation of the precipitated [^{3}H]palmitate-labeled HSPG with 3.3 μg/ml of PI-PLC for 2 h at 37°C resulted in a significant removal of the label as compared to incubation with medium alone (results not shown). This indicated that part of the fatty acid was incorporated into the PI anchor. The HSPG was not precipitated by addition of protein A-Sepharose beads coated with preimmune rabbit IgG (not shown). Bone marrow cells were noted to express two or three other bFGF-binding proteins that were labeled by palmitic acid as these were also specifically precipitated with bFGF and anti-bFGF IgG (lane *2*). This might be due to the limited specificity of the metabolic labeling using fatty acids, since they have been found to be covalently attached to proteins by direct acylation (Schmidt, 1989) or to be converted to amino acids.

The release of the 200-kD HSPG by low concentrations of PI-PLC, as well as the presence of ethanolamine and a PI-PLC-removable fatty acid, indicate cell surface linkage of this molecule via a PI anchor.

Discussion

The multifunctional, autocrine growth factor, bFGF, is known to be a potent mitogen for human bone marrow stromal cells (Oliver et al., 1990) and also stimulates myelopoiesis in long-term human bone marrow cultures (Wilson et al., 1991). In this report, we have shown that bFGF can be extracted from the ECM of primary human bone marrow cultures, as has been demonstrated for endothelial cell-derived ECM (Vlodavsky et al., 1987). This suggests that bFGF is produced by human bone marrow cells and that it is subsequently deposited into the stromal cell matrix. We

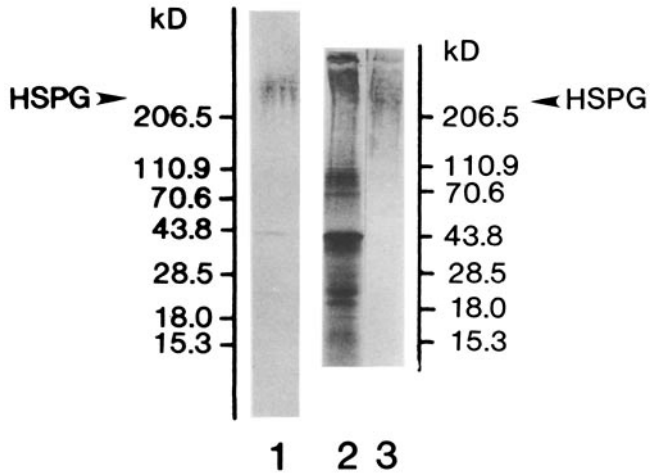

Figure 8. Metabolic labeling of the PI anchor of the 200-kD HSPG. Cells were incubated overnight with 167 μCi/ml of [^{3}H]ethanolamine (lane *1*), 0.8 mCi/ml of a [^{3}H]palmitic acid-BSA complex (lane *2*), or with 40 μCi/ml of [^{35}S]sulfate (lane *3*) (see Materials and Methods). Cell extracts were prepared as described in Fig. 1. and bFGF-binding HSPGs were isolated by addition of protein A-Sepharose coated with bFGF-anti-bFGF IgG complexes. Precipitates were analyzed by SDS-PAGE followed by autoradiography as described in Fig. 5. Addition of protein A–Sepharose beads coated with preimmune rabbit IgG did not precipitate any of these bands (not shown). The results of two separate labeling experiments are shown.

were, however, unable to detect significant amounts of bFGF in Triton X-100 cell lysates of bone marrow cells. This could be explained by the observation that the bFGF released during cell lysis is absorbed by the ECM (Gajdusek and Carbon, 1989). An alternative explanation is the limited sensitivity of the immunoblotting technique. Thus, the concentration of bFGF in cell lysates might have been too low to be detected, whereas continuous bFGF deposition in the ECM probably led to the enrichment of the growth factor in the matrix.

Primary bone marrow cultures had a high capacity for binding bFGF (>70 ng/10^5 cells), and their binding sites were not saturable by concentrations as high as 1 μg/ml of growth factor. More than 95% of the bFGF binding sites in bone marrow cultures were heparin-like in nature, which is consistent with earlier results obtained with BCE cells, which express >10^6 heparin-like bFGF binding sites per cell compared to only 6,000–17,000 high affinity bFGF receptors per cell (Moscatelli, 1987, 1988).

The major bFGF binding protein in primary human bone marrow cultures was characterized as a cellular HSPG with a molecular size of ~200 kD. This species was found in cell lysates and in conditioned media. The size of this bFGF-binding HSPG is similar to one of the bFGF-binding HSPGs of BCE cells identified previously (Saksela and Rifkin, 1990), which had a molecular mass of ~250 kD. Both proteoglycans were found in cell lysates and conditioned medium.

bFGF was very efficiently released from its binding sites in human bone marrow cultures by incubation with PI-PLC. Equimolar amounts of plasmin and heparinase were either less efficient or unable to solubilize significant amounts of bFGF in this system. However, it should be noted that the catalytic efficiencies of the different enzyme preparations were unknown. The bFGF was solubilized by PI-PLC as a complex with the 200-kD HSPG, since both molecules were coprecipitated with anti-bFGF IgG. This complex exhibited biological activity as it stimulated PA production in BAE cells, supporting the hypothesis that bFGF-HSPG complexes are biologically active and relevant. In fact, Yayon et al. (1991) have recently shown that heparin-like molecules are essential for the binding of bFGF to its high affinity receptor.

Essentially complete release of the major bFGF-binding molecule, the 200-kD HSPG (constituting ~40% of the total cellular proteoglycan), was observed after exposure to PI-PLC. Plasmin was less effective in solubilizing the 200-kD HSPG. The solubilization of the 200-kD HSPG by PI-PLC indicated a linkage of this proteoglycan to the cell surface via a PI anchor. This was confirmed by the metabolic labeling of the HSPG with [^{3}H]ethanolamine or [^{3}H]palmitic acid. The fatty acid label could be partially removed by treatment of the HSPG with PI-PLC, indicating incorporation of the fatty acid in the PI anchor. PI-linked HSPGs have been described for several other cell types, hepatocytes (Ishihara et al., 1987), Schwann cells (Carey and Evans, 1989), ovarian granulosa cells (Yanagashita and McQuillan, 1989), and lung fibroblasts (David et al., 1990). In contrast, a significant release by PI-PLC of the bFGF-binding HSPGs of BAE and BCE cells could not be detected under the conditions used. However, since the experiments with the bone marrow cultures were performed with primary human cells, while the experiments with the bovine endothelial cells were not, the presence of PI-linked HSPGs on primary human endothelial cells can not be excluded. In addition, the PI anchors in different PI-linked proteins vary in their structure and not all of these molecules can be released by PI-PLC (Low, 1989).

Despite the large variety of proteins found to be linked to the cell surface via PI anchors (Low, 1989), the biological significance of this anchor and the mechanism of its cleavage in vivo are not yet understood. In the instance of the 200-kD bFGF-binding HSPG of human bone marrow cultures, the PI linkage might provide a highly selective and efficient mechanism for releasing active growth factor without any additional perturbation of the microenvironment. Furthermore, the biologically active bFGF-HSPG complex, together with other PI-linked molecules on the stromal cell surface, might comprise a group of factors, which can cooperate in supporting haematopoiesis and which can be made available by the action of a single specific enzyme. While a constitutive secretion or shedding of the 200-kD HSPG from the bone marrow cultures can not be excluded, its spontaneous release even in the presence of the plasmin inhibitor aprotinin might indicate the presence of an endogenous anchor-specific phospholipase in these cultures.

This novel mechanism of the phospholipase-catalyzed release of active bFGF in human bone marrow cultures might result in the specific and sensitive regulation of the hematopoietic process in vivo. A cell surface–bound PI-PLC has recently been detected on Swiss 3T3 fibroblasts (Ting and Pagano, 1990), and anchor-specific phospholipase Ds have been identified and purified from human plasma and bovine brain (Davitz et al., 1989; Hoener et al., 1990). Moreover, an anchor-specific phospholipase D has been demonstrated in human mast cells, a cell type which originates in the bone

marrow (Gleichauf, C., P. Thomas, and M. A. Davitz. 1990. *J. Cell Biol.* 111:1098). However, an enzyme that might be capable of catalyzing the release of bFGF-HSPG complexes in human bone marrow has not yet been identified.

In conclusion, bFGF deposition in the microenvironment of bone marrow stromal cells and its release in a biologically active form either by a specific phospholipase or by the initiation of the proteolytic cascade of plasminogen activation, might constitute important events in cell–cell interactions of stromal and progenitor cells during haematopoiesis.

The authors would like to thank P. A. Dennis for providing affinity-purified anti-bFGF IgG, Dr. D. Moscatelli for the kind gift of [^{125}I]bFGF, and Dr. M. A. Davitz for his helpful advice.

This work was supported by grant No. Br 1125/1-1 from the Deutsche Forschungsgemeinschaft (DFG), by grants from the Leukemia Society of America, by grants No. CA 49419, CA 34282, and CA 20194 from the National Institutes of Health, Bethesda, MD, the Benjamin Weiss Fund, the South African National Cancer Association, and the University of Cape Town Staff Research Fund.

Received for publication 24 January 1991 and in revised form 24 May 1991.

References

Brunner, G., J. Pohl, L. J. Erkell, A. Radler-Pohl, and V. Schirrmacher. 1989. Induction of urokinase activity and malignant phenotype in bladder carcinoma cells after transfection of the activated Ha-ras oncogene. *J. Cancer Res. Clin. Oncol.* 115:139–144.

Carey, D. J., and D. M. Evans. 1989. Membrane anchoring of heparan sulfate proteoglycans by phosphatidylinositol and kinetics of synthesis of peripheral and detergent-solubilized proteoglycans in Schwann cells. *J. Cell Biol.* 108:1891–1897.

Clark, S. C., and R. Kamen. 1987. The human hematopoietic colony-stimulating factors. *Science (Wash. DC).* 236:1229–1237.

David, G., V. Lories, B. Decock, P. Marynen, J. J. Cassiman, and H. Van den Berghe. 1990. Molecular cloning of a phosphatidylinositol-anchored membrane heparan sulfate proteoglycan from human lung fibroblasts. *J. Cell Biol.* 111:3165–3176.

Davitz, M. A., J. Hom, and S. Schenkman. 1989. Purification of a glycosylphosphatidylinositol-specific phospholipase D from human plasma. *J. Biol. Chem.* 264:13760–13764.

Dennis, P. A., and D. B. Rifkin. 1990. Studies on the role of basic fibroblast growth factor in vivo: inability of neutralizing antibodies to block tumor growth. *J. Cell. Physiol.* 144:84–98.

Deutsch, D. G., and E. T. Mertz. 1970. Plasminogen: purification from human plasma by affinity chromatography. *Science (Wash. DC).* 170:1095–1096.

Dexter, T. M. 1982. Stromal cell associated haemopoiesis. *J. Cell. Physiol. Suppl.* 1:87–94.

Dexter, T. M., T. D. Allen, and L. G. Lajtha. 1977. Conditions controlling the proliferation of haemopoietic stem cells in vitro. *J. Cell. Physiol.* 91:335–344.

Flaumenhaft, R., D. Moscatelli, O. Saksela, and D. B. Rifkin. 1989. Role of extracellular matrix in the action of basic fibroblast growth factor: matrix as a source of growth factor for long-term stimulation of plasminogen activator production and DNA synthesis. *J. Cell. Physiol.* 140:75–81.

Flaumenhaft, R., D. Moscatelli, and D. B. Rifkin. 1990. Heparin and heparan sulfate increase the radius of diffusion and action of basic fibroblast growth factor. *J. Cell Biol.* 111:1651–1659.

Folkman, J., C. C. Haudenschild, and B. R. Zetter. 1979. Long-term culture of capillary endothelial cells. *Proc. Natl. Acad. Sci. USA.* 76:5217–5221.

Gabbianelli, M., M. Sargiacomo, E. Pelosi, U. Testa, G. Isacchi, and C. Peschle. 1990. "Pure" human hematopoietic progenitors: permissive action of basic fibroblast growth factor. *Science (Wash. DC).* 249:1561–1564.

Gajdusek, C. M., and S. Carbon. 1989. Injury-induced release of basic fibroblast growth factor from bovine aortic endothelium. *J. Cell. Physiol.* 139: 570–579.

Gauthier, T., M. Maftouh, and C. Picard. 1987. Rapid enzymatic degradation of [^{125}I](tyr 10)FGF(1-10) by serum *in vitro* and involvement in the determination of circulating FGF by RIA. *Biochem. Biophys. Res. Commun.* 145:775–781.

Gordon, M. Y., G. P. Riley, S. M. Watt, and M. F. Greaves. 1987. Compartmentalization of a haematopoietic growth factor (GM-CSF) by glycosaminoglycans in the bone marrow microenvironment. *Nature (Lond.).* 326:403–405.

Hoener, M. C., S. Stieger, and U. Brodbeck. 1990. Isolation and characterization of a phosphatidylinositol-glycan-anchor-specific phospholipase D from bovine brain. *Eur. J. Biochem.* 190:593–601.

Ishai-Michaeli, R., A. Eldor, and I. Vlodavsky. 1990. Heparanase activity expressed by platelets, neutrophils, and lymphoma cells releases active fibroblast growth factor from extracellular matrix. *Cell Regulation.* 1:833–842.

Ishihara, M., N. S. Fedarko, and H. E. Conrad. 1987. Involvement of phosphatidylinositol and insulin in the coordinate regulation of proteoheparan sulfate metabolism and hepatocyte growth. *J. Biol. Chem.* 262:4708–4716.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

Low, M. G. 1989. The glyco-phosphatidylinositol anchor of membrane proteins. *Biochim. Biophys. Acta.* 988:427–454.

Metcalf, D. 1986. The molecular biology and function of the granulocyte-macrophage colony-stimulating factors. *Blood.* 67:257–267.

Moscatelli, D. 1987. High and low affinity binding sites for basic fibroblast growth factor on cultured cells: absence of a role for low affinity binding in the stimulation of plasminogen activator production by bovine capillary endothelial cells. *J. Cell. Physiol.* 131:123–130.

Moscatelli, D. 1988. Metabolism of receptor-bound and matrix-bound basic fibroblast growth factor by bovine capillary endothelial cells. *J. Cell Biol.* 107:753–759.

Moscatelli, D., and P. Devesly. 1990. Turnover of functional basic fibroblast growth factor receptors on the surface of BHK and NIH 3T3 cells. *Growth Factors.* 3:25–33.

Moscatelli, D., M. Presta, and D. B. Rifkin. 1986. Purification of a factor from human placenta that stimulates capillary endothelial cell protease production, DNA synthesis, and migration. *Proc. Natl. Acad. Sci. USA.* 83:2091–2095.

Oliver, L. J., D. B. Rifkin, J. Gabrilove, M. J. Hannocks, and E. L. Wilson. 1990. Long-term culture of human bone marrow stromal cells in the presence of basic fibroblast growth factor. *Growth Factors.* 3:231–236.

Presta, M., D. Moscatelli, J. Joseph-Silverstein, and D. B. Rifkin. 1986. Purification from a human hepatoma cell line of a basic fibroblast growth factor-like molecule that stimulates capillary endothelial cell plasminogen activator production, DNA synthesis, and migration. *Mol. Cell. Biol.* 6:4060–4066.

Rifkin, D. B., and D. Moscatelli (1989). Recent developments in the cell biology of basic fibroblast growth factor. *J. Cell Biol.* 109:1–6.

Roberts, R., J. Gallagher, E. Spooncer, T. D. Allen, F. Bloomfield, and T. M. Dexter. 1988. Heparan sulphate bound growth factors: a mechanism for stromal cell mediated haemopoiesis. *Nature (Lond.).* 332:376–378.

Saksela, O., and D. B. Rifkin. 1990. Release of basic fibroblast growth factor-heparan sulfate complexes from endothelial cells by plasminogen activator-mediated proteolytic activity. *J. Cell Biol.* 110:767–775.

Saksela, O., D. Moscatelli, A. Sommer, and D. B. Rifkin. 1988. Endothelial cell-derived heparan sulfate binds basic fibroblast growth factor and protects it from proteolytic degradation. *J. Cell Biol.* 107:743–751.

Sato, Y., and D. B. Rifkin. 1988. Autocrine activities of basic fibroblast growth factor: regulation of endothelial cell movement, plasminogen activator synthesis, and DNA synthesis. *J. Cell Biol.* 107:1199–1205.

Schmidt, M. F. G. 1989. Fatty acylation of proteins. *Biochim. Biophys. Acta.* 988:411–426.

Schor, A. M., and S. L. Schor. 1988. Inhibition of endothelial cell morphogenetic interactions in vitro by alpha- and beta-xylosides. *In Vitro (Rockville).* 24:659–668.

Sommer, A., M. T. Brewer, R. C. Thompson, D. Moscatelli, M. Presta, and D. B. Rifkin. 1987. A form of human basic fibroblast growth factor with an extended amino terminus. *Biochem. Biophys. Res. Commun.* 144: 543–550.

Ting, A. E., and R. E. Pagano. 1990. Detection of a phosphatidylinositol-specific phospholipase C at the surface of Swiss 3T3 cells and its potential role in the regulation of cell growth. *J. Biol. Chem.* 265:5337–5340.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.

Vlodavsky, I., J. Folkman, R. Sullivan, R. Fridman, R. Ishai-Michaeli, J. Sasse, and M. Klagsbrun. 1987. Endothelial cell-derived basic fibroblast growth factor: synthesis and deposition into subendothelial extracellular matrix. *Proc. Natl. Acad. Sci. USA.* 84:2292–2296.

Wilson, E. L., D. B. Rifkin, F. Kelly, M. J. Hannocks, and J. L. Gabrilove. 1991. Basic fibroblast growth factor stimulates myelopoiesis in long-term human bone marrow cultures. *Blood.* 77:954–960.

Yanagashita, M., and D. J. McQuillan. 1989. Two forms of plasma membrane-intercalated heparan sulfate proteoglycans in rat ovarian granulosa cells. *J. Biol. Chem.* 264:17551–17558.

Yayon, A., M. Klagsbrun, J. D. Esko, P. Leder, and D. M. Ornitz. 1991. Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. *Cell.* 64:841–848.