Release of Basic Fibroblast Growth Factor–Heparan Sulfate Complexes from Endothelial Cells by Plasminogen Activator–mediated Proteolytic Activity

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Abstract. Cultured bovine capillary endothelial (BCE) cells synthesize heparan sulfate proteoglycans (HSPG), which are both secreted into the culture medium and deposited in the cell layer. The nonsoluble HSPGs can be isolated as two predominant species: a larger 800-kD HSPG, which is recovered from preparations of extracellular matrix, and a 250-kD HSPG, which is solubilized by nonionic detergent extraction of the cells. Both HSPG species bind bFGF. ^{125}I-bFGF bound to BCE cell cultures is readily released by either heparinase or plasmin. When released by plasmin, the growth factor is recovered from the incubation medium as a complex with the partly degraded high molecular mass HSPG. Endogenous bFGF activity is released by a proteolytic treatment of cultured BCE cells.

The bFGF-binding HSPGs are also released when cultures are incubated with the inactive proenzyme plasminogen. Under such experimental conditions, the release of the extracellular proteoglycans can be enhanced by treating the cells either with bFGF, which increases the plasminogen activating activity expressed by the cells, or decreased by treating the cells with transforming growth factor beta, which decreases the plasminogen activating activity of the cells. Specific immune antibodies raised against bovine urokinase also block the release of HSPG from BCE cell cultures. We propose that this plasminogen activator–mediated proteolysis provides a mechanism for the release of biologically active bFGF–HSPG complexes from the extracellular matrix and that bFGF release can be regulated by the balance between factors affecting the pericellular proteolytic activity.

Basic fibroblast growth factor (bFGF) is a multifunctional growth factor present in several tissues in vivo and is synthesized by a number of cell types in vitro (15, 32). It affects the growth and differentiation of many cells of ectodermal or mesodermal origin via a high affinity membrane receptor (29, 30, 35, 36). bFGF is a potent endothelial growth factor in vitro and is capable of inducing angiogenesis in vivo at nanogram amounts (21). It is thought to function as an autocrine factor in cultures of endothelial cells (46, 48).

Pathways for the release of bFGF have so far remained uncharacterized. The precursor molecule does not have a classical signal sequence which would mediate its secretion into the extracellular space (1). However, bFGF is recovered from extracellular matrix both in vitro and in vivo (2, 12, 49, 52) and can be released from its binding sites by heparinase digestion or by trypsin (3, 52). Extracellular matrix may serve as a reservoir for bFGF since binding to matrix structures is responsible for the long-term stimulation of endothelial cells even after a short exposure to the growth factor (10).

bFGF is characterized by its high affinity towards heparin and has been shown to bind to heparan sulfates secreted into the growth medium by cultured endothelial cells (43). Heparan sulfate is an abundant component of most extracellular structures and it has been suggested to play a role in cellular interactions and in growth control during morphogenesis, cell differentiation, and growth (5, 19, 45, 51). Cultured aortic endothelial cells synthesize three different types of heparan sulfate: a cell membrane–associated heparan sulfate proteoglycan (HSPG),[1] an extracellular matrix-associated HSPG, and a small heparan sulfate found only in the culture medium (23, 24). The protein core of the cell-associated HSPGs is sensitive to degradation by wide-spectrum serine proteinases such as trypsin. Degradation of the core protein leads to the release of heparan sulfate chains still bound to proteinase-resistant core protein peptides (38).

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1. *Abbreviations used in this paper*: BCE, bovine capillary endothelial; HSPG, heparan sulfate proteoglycan; KIU, Kallikrein inhibitor units; PA, plasminogen activator; PAI-1, type 1 plasminogen activator inhibitor; TGFβ, transforming growth factor β; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator.

A widely distributed enzyme with a substrate specificity much like that of trypsin is plasmin, which is formed from the inactive precursor molecule plasminogen by proteolytic activation (40). Two separate serine proteinases capable of effectively activating plasminogen have been characterized. They are the urokinase-type plasminogen activator (uPA), usually associated with cell migration and tissue destruction, and the tissue-type plasminogen activator (tPA), associated with thrombolysis and possibly prohormone activation and release (6). The activity of plasminogen activators (PAs) is partly regulated by highly specific proteinase inhibitors expressed in vitro by a variety of cell types (41). The principal inhibitor released by endothelial cells is the type 1 plasminogen activator inhibitor (PAI-1), which reacts rapidly with both uPA and tPA. Due to the high levels of plasmin inhibitors found in the circulation and in most extracellular fluids it is likely that plasmin function is restricted to sites where the active enzyme is unavailable to its soluble inhibitors and where PA is not subject to inactivation by its inhibitor (PAI-1). Therefore, the modulation of the local release of PA and its inhibitor may be the crucial step in the regulation of plasmin-mediated proteolysis.

In the present article we propose a role for the plasminogen activation cascade in the release of matrix-bound bFGF from the heparan sulfate–rich extracellular milieu of capillary endothelial cells. The soluble bFGF is found as a complex with HSPG. The release of bFGF-binding HSPG complexes can be modulated by the PA activity expressed by the cells themselves. The findings indicate a primary role for PA and its inhibitor in mediating matrix breakdown, which in turn may affect the release of matrix-bound growth factors.

Materials and Methods

Reagents

bFGF was either purified from full-term human placentas (33, 37) or was prepared by recombinant techniques in *Escherichia coli* and was a gift from Dr. A. Sommer (Synergen Inc., Boulder, CO) (50). Both preparations gave identical results with respect to their ability to bind heparan sulfate. Plasminogen was purified from outdated human plasma as described elsewhere (7). Plasmin, trypsin, heparinase, chondroitinase ABC, methylumberyl-liferum-b-xyloside, heparin, guanidine, urea, and protein A-Sepharose were obtained from Sigma Chemical Co. (St. Louis, MO) and were the highest grade available. DEAE-cellulose (DE-52) was from Whatman Inc. (Clifton, NJ). Antibodies against bFGF were raised in rabbits and their specificity was confirmed as described earlier (22, 32). These antibodies recognize bFGF but not other heparin-binding growth factors. Antibodies against bovine urokinase were raised in rabbits by injecting the animals with urokinase purified from cultures of bovine kidney epithelial cells. IgG was further purified from the serum by affinity chromatography on protein G-Sepharose according to the manufacturer's procedure (Pharmacia Fine Chemicals, Uppsala, Sweden). The IgG preparation at a concentration of 500 μg/ml completely inhibited the urokinase-dependent activity produced by 10^5 bovine capillary endothelial (BCE) cells placed in contact with radiolabeled fibrin substrate for 4 h in plasminogen-containing medium. The antibodies only partially neutralized human urokinase activity. Polyclonal antibodies against the human uPA and tPA were obtained from American Diagnostica (New York, NY).

Cell Cultures

BCE cells were isolated from the adrenal cortex by the method of Folkman et al. (11), and the cloned cells were identified as endothelial cells by staining for factor VIII–related antigen (11). The cells were grown in alpha-modified MEM supplemented with 5% newborn calf serum and antibiotics and used for experiments between passages 6 and 15. When indicated, the cell cultures were labeled with $^{35}SO_4$ (40 μCi/ml; New England Nuclear, Boston, MA) for 16 h in serum-free alpha-modified MEM.

Isolation of the Metabolically Labeled Proteoglycans

$^{35}SO_4$-labeled cells were extracted with 4 M guanidine-HCl in 50 mM Na-acetate buffer, pH 6.0, containing 2% Triton X-100, 1 mM PMSF, and 200 Kallikrein inhibitor units (KIU)/ml aprotinin (17). After a 6-h incubation at 4°C, the extract was applied to a column of Sepharose G-25 (0.6 × 10 cm) equilibrated with 8 M urea in 50 mM Na-acetate buffer, pH 6.0, containing 0.5% Triton X-100, 0.15 M NaCl, PMSF, and aprotinin and eluted from the column with the same buffer. Fractions containing the radioactivity were pooled and bound to a DE-52 column (0.5 × 2 cm) equilibrated with the 8 M urea buffer. The bound radioactivity was eluted with a 0.15–1.0 M NaCl gradient in the above buffer. Labeled material eluting between 0.20 and 0.75 M NaCl was pooled and used for further experiments as described in the text.

Binding of the Isolated $^{35}SO_4$-labeled Proteoglycans to bFGF

bFGF was added to samples of the isolated proteoglycans and incubated at 37°C for 15 min. Complexes that formed were precipitated by adding rabbit anti-bFGF IgG immobilized on protein A–Sepharose beads. The pellet was washed with PBS (0.01 M PO_4, 0.15 M NaCl, pH 7.4), and the bound molecules were eluted with reducing sample buffer. The samples were analyzed by electrophoresis in a 3–16% SDS-polyacrylamide slab gel (25), and the radiolabeled molecules were visualized by autoradiography.

Xyloside Treatment of BCE Cells

Cells were trypsinized and grown in the presence of 1.0 mM methylumberyl-liferum-b-xyloside in alpha-modified MEM supplemented with 5% calf serum (47). Fresh medium containing the xyloside was added to the cells twice a week. The xyloside was added to the medium during overnight labeling of the cells. The growth rate and morphology of BCE cells in the presence of the xyloside were comparable with those seen in the control cultures.

Binding of ^{125}I-bFGF to BCE Cells

bFGF was iodinated as described by Moscatelli (29). ^{125}I-bFGF (specific activity 1,100 cpm/fmol) mixed with cold bFGF in PBS containing 10 mM Hepes and 0.15% gelatin was added to BCE cells grown on 35-mm dishes and incubated at 4°C for 30 min. Unbound bFGF was removed, and the cells were washed thrice with cold PBS. The cell cultures were treated as described in the text.

Triton X-100 Extraction of BCE Cells and Preparation of the Extracellular Matrix

Radiolabeled cultures were washed twice with cold PBS and treated with 0.5% Triton X-100 in 10 mM Tris-HCl buffer, pH 7.4. Extraction of the cells was carried out at 4°C for 30 min on a shaker. The Triton X-100 extract was collected, and insoluble material was removed by centrifugation at 10,000 *g* for 10 min. The dishes were further incubated at 4°C with the above detergent solution containing 20 mM NH_4OH, washed thrice with PBS, and used for experiments as indicated in the text. Both detergent solutions contained 2 mM PMSF and 200 KIU/ml aprotinin.

PA Assays

Cell-associated PA activity was measured in cells incubated for 16 h in serum-free medium and then extracted in 0.5% Triton X-100 in 0.1 M Tris-HCl, pH 8.1. Activity was quantitated by the ^{125}I-fibrin plate assay as described (16) and expressed in Ploug units using purified urokinase (Calbiochem-Behring Corp., La Jolla, CA) as a standard.

Results

Isolation of bFGF-binding HSPG from BCE Cells

We have previously demonstrated that cultured BCE cells

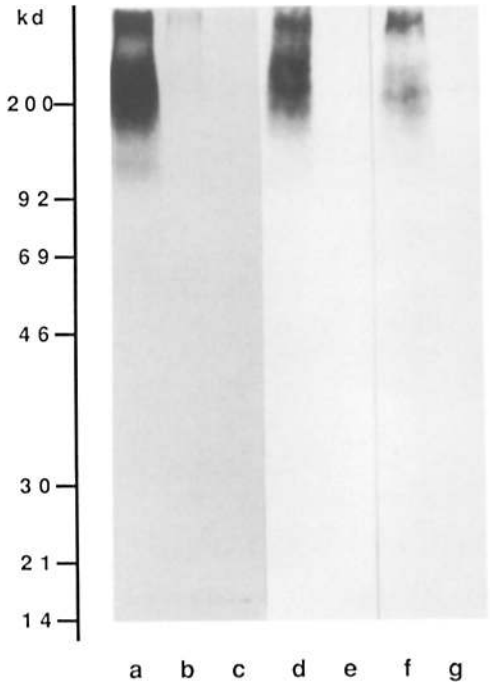

Figure 1. Isolation of $^{35}SO_4$-labeled bFGF-binding molecules from extracts of BCE cells. Cultures of confluent cells were labeled overnight, and bFGF-binding molecules were isolated as described in Materials and Methods and analyzed in SDS-PAGE followed by autoradiography (lane *a*). (Lane *b*) Material bound to anti-bFGF IgG without addition of bFGF; (lane *c*) material bound to protein A-Sepharose particles alone; (lanes *d* and *e*) material remaining after bound molecules were treated with chondroitinase ABC or heparinase, respectively; (lane *f*) molecules recovered from conditioned medium of BCE cells by absorption with antibody-immobilized bFGF; (lane *g*) heparinase digestion of material from lane *f*.

synthesize and release into the growth medium HSPGs which bind bFGF (43). To study further the origin and metabolism of these soluble bFGF-binding molecules, we isolated HSPGs from the cell layer and characterized the mechanisms involved in their release.

BCE cell cultures labeled with $^{35}SO_4$ were extracted with guanidine, and the extracts were changed to urea by chromatography through a Sepharose G-25 column, bound to DE-52, and eluted with a salt gradient. Most of the radioactivity eluted between 0.25 and 0.75 M NaCl (not shown). The eluted radiolabeled material was pooled, and the sample was adjusted with water to give final concentrations of 0.16 M NaCl and 2.7 M urea. The glycosaminoglycans were bound to bFGF by adding the growth factor to a sample of the above eluate. The complexes of HSPG and bFGF which formed were precipitated with anti-bFGF immobilized on protein A-Sepharose and analyzed by SDS-PAGE (Fig. 1, lane *a*). Two bands of radioactivity were observed, one corresponding to a molecule with a molecular mass of 800 kD and the second at a position corresponding to 250 kD. Barely detectable amounts of the $^{35}SO_4$-labeled material bound to anti-bFGF beads without prior addition of bFGF to the samples (Fig. 1, lane *b*) or to protein A-Sepharose particles alone (Fig. 1, lane *c*). To demonstrate the nature of the bound $^{35}SO_4$-labeled molecules, protein A-Sepharose beads containing the bFGF-glycosaminoglycan complexes were dispersed in PBS, and the samples were treated with 2 IU/ml of chondroitinase ABC (Fig. 1, lane *d*) or 2 IU/ml of heparinase (Fig. 1, lane *e*) for 3 h at 37°C in the presence of 200 KIU/ml of aprotinin. After the incubation, sample buffer was added, and the material was analyzed by SDS-PAGE. The results demonstrate that all bFGF-bound $^{35}SO_4$-labeled molecules were degraded by heparinase but not by chondroitinase ABC.

As shown previously (43), molecules of a similar size and sensitive to heparinase digestion can be recovered from the conditioned medium of these cultures by direct absorption with antibody-immobilized bFGF (Fig. 1, lanes *f* and *g*).

Localization of the bFGF-binding Molecules in Cultures of BCE Cells

To determine the cellular localization of the two bFGF-binding heparan sulfates, the cells were extracted with 0.5% Triton X-100. This procedure removes the cytoplasmic and membrane structures and leaves a well-characterized complex of extracellular matrix components bound to the growth substratum (18). The molecules that remain associated with the matrix can, in turn, be solubilized with guanidine. $^{35}SO_4$-labeled BCE cell cultures were separated into Triton X-100–soluble and -insoluble fractions, and the insoluble

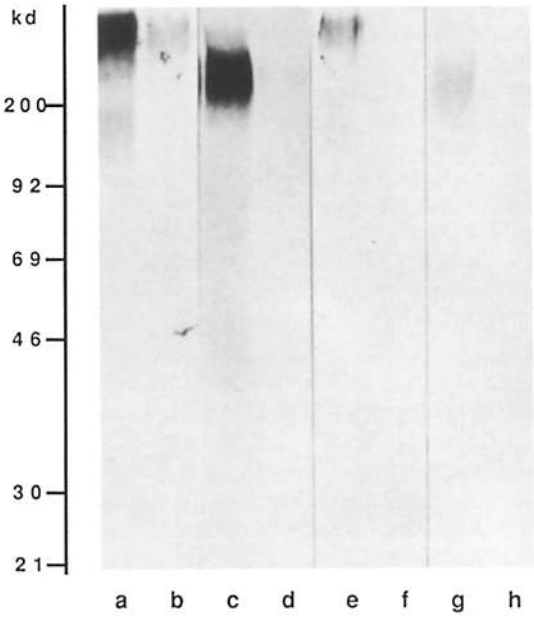

Figure 2. Demonstration of the localization of the bFGF-binding HSPGs. $^{35}SO_4$-labeled cultures of BCE cells were treated with Triton X-100 and guanidine-urea as described in Materials and Methods, and the solubilized, radiolabeled bFGF-binding molecules were analyzed in SDS-PAGE followed by autoradiography. (Lane *a*) Guanidine-urea extract of isolated matrix; (lane *c*) Triton X-100 extract of cells; (lanes *b* and *d*) material isolated as in lanes *a* and *c*, respectively, but after heparinase treatment of intact cells before extraction of the cultures; (lanes *e* and *g*) material isolated as in lanes *a* and *c*, respectively, from cultures grown in the presence of methylumberylliferum-beta-xyloside; (lanes *f* and *h*) material isolated from methylumberylliferum-beta-xyloside-treated cells as in lanes *e* and *g*, respectively, but after heparinase pretreatment of the cells.

fraction was eluted with guanidine as described for the whole cell extraction. The radiolabeled molecules from the Triton X-100 extract and from the guanidine-urea extract were then bound to DE-52, eluted with 0.5 M NaCl, bound to immobilized bFGF, and analyzed by SDS-PAGE (Fig. 2). The high molecular mass 800-kD heparan sulfate was recovered from the Triton X-100–insoluble fraction, whereas the smaller 250-kD heparan sulfate was solubilized by the Triton X-100 treatment. (Fig. 2, lanes *a* and *c*).

To demonstrate the extracellular nature of both of the molecules, 5 IU/ml of heparinase was added to the cells for the last 3 h of metabolic labeling. After the labeling, the cells were washed thrice with cold PBS to remove the enzyme, and separated into Triton X-100–soluble and -insoluble fractions by adding cold 0.5% Triton X-100 at 4°C as described. The insoluble molecules were then extracted with 4 M guanidine, and the bFGF-binding molecules were purified and characterized as above. The heparinase treatment resulted in the disappearance of both the Triton X-100–insoluble and -soluble heparan sulfates (Fig. 2, lanes *b* and *d*). These results suggest that the majority of the smaller bFGF-binding heparan sulfate is located at the exterior of the cell in a Triton X-100–extractable, heparinase-sensitive form. The larger $^{35}SO_4$-labeled molecule appears to be a component of the extracellular matrix and can also be degraded by the added heparinase.

The 800-kD bFGF-binding heparan sulfate located both in the conditioned medium (43) and in the cell extract (not shown) could be labeled with [^{35}S]methionine, thus demonstrating the presence of a protein core in the molecule, whereas the smaller heparan sulfate could not be labeled with [^{35}S]methionine (not shown). To demonstrate that both of the bFGF-bound heparan sulfates were proteoglycans or were derived from proteoglycans, BCE cells were grown in the presence of beta-xylosides, which can act as primers for the synthesis of the proteoglycan polysaccharide side chains. The enzyme xylosyltransferase is responsible for the addition of the first sugar to the proteoglycan core protein during synthesis of the polysaccharide side chains (47). The presence of beta-xylosides leads to synthesis of free glycosaminoglycan chains. Addition of 1 mM methylumberylliferum-beta-xyloside to the growth medium of the cells resulted in an almost total disappearance of both of the bFGF-binding high molecular mass heparan sulfate species from the Triton X-100–soluble and -insoluble fractions (compare Fig. 2, lanes *e* and *g*, with lanes *a* and *c*, which were obtained in the absence of xylosides).

Release of the bFGF-binding HSPG by Plasmin Digestion

The sensitivity of the bFGF-binding extracellular HSPGs to release by proteolysis was tested by adding increasing amounts of plasmin (final concentrations 0.1, 1, and 10 μg/ml) to cultures of BCE cells. After a 20-min incubation, the medium was collected, plasmin activity was inhibited by addition of aprotinin (100 KIU/ml), and the released molecules were bound to immobilized bFGF. As shown in Fig. 3, lanes *a–c*, bFGF-binding $^{35}SO_4$-labeled HSPGs were released from the cells under these conditions. The high molecular mass species had a slightly smaller molecular mass than the corresponding bFGF-binding molecule from the whole cell extracts or from the matrix preparations.

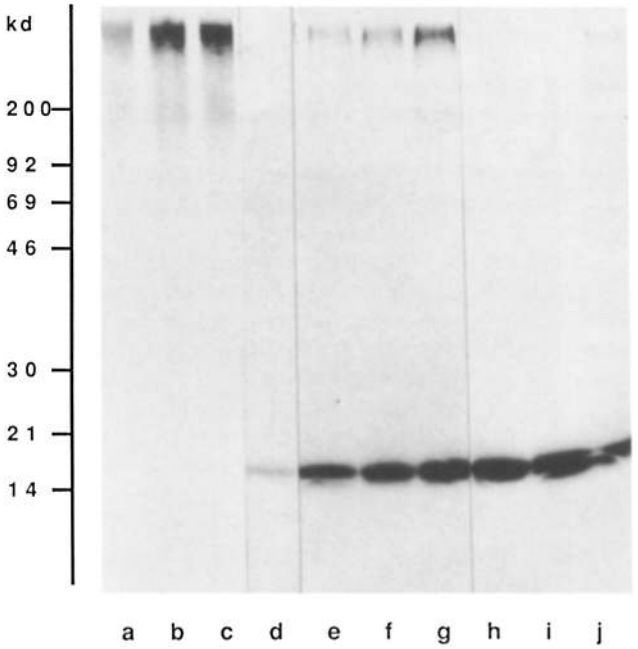

Figure 3. Release of bFGF–HSPG complexes by plasmin digestion. FGF-binding HSPGs were isolated as described in Materials and Methods after plasmin treatment of $^{35}SO_4$-labeled cells and analyzed by SDS-PAGE followed by autoradiography. (Lane *a*) 0.1 μg/ml plasmin; (lane *b*) 1 μg/ml plasmin; (lane *c*) 10 μg/ml plasmin. (Lanes *d–g*) Release by plasmin of ^{125}I-bFGF from cultures of BCE cells: (lane *d*) no plasmin; (lane *e*) 0.1 μg/ml plasmin; (lane *f*) 1 μg/ml plasmin; (lane *g*) 10 μg/ml plasmin. (Lanes *h–j*) Release of bound ^{125}I-labeled bFGF by heparinase treatment: (lane *h*) 0.1 IU/ml heparinase; (lane *i*) 1 IU/ml heparinase; (lane *j*) 10 IU/ml heparinase. Material in lanes *d–j* was concentrated by binding to protein A–immobilized anti–bFGF IgG.

Release of ^{125}I-bFGF from BCE by Plasmin and Heparinase Treatment

To test whether plasmin could release the cell-bound bFGF as a complex with HSPG, the following experiment was performed. 5 μg/ml of bFGF mixed with 10,000 cpm/ml ^{125}I-bFGF (specific activity 1,100 cpm/fmol) was bound to $^{35}SO_4$-labeled BCE cells grown on 35-mm dishes as described in Materials and Methods. After washing the cells several times with PBS to remove unbound growth factor, the cells were incubated with increasing concentrations of plasmin (0.1, 1, and 10 μg/ml) in PBS for 20 min at 37°C. After the incubation, plasmin activity was inhibited by addition of aprotinin. The released material was absorbed by anti–bFGF IgG immobilized on protein A–Sepharose particles and analyzed by SDS-PAGE. As shown in Fig. 3, lanes *e–g*, bFGF was rapidly released from the cell culture with low concentrations of plasmin. The precipitation of the plasmin-released bFGF with anti–bFGF IgG resulted in coprecipitation of the high molecular mass $^{35}SO_4$-labeled HSPG. The fact that the bFGF was not degraded under these conditions is consistent with our earlier observation that bFGF complexed to HSPG is resistant to proteolysis by trypsin or plasmin (43). Heparinase (0.1, 1, and 10 IU/ml) also readily released the cell-bound bFGF. However, no $^{35}SO_4$-labeled molecules were detectable in the SDS-PAGE analysis of the heparinase releasate (Fig. 3, lanes *h–j*).

Demonstration of Endogenous bFGF Activity in the Protease-released Material from BCE Cells

Although the uncomplexed bFGF is readily degraded by proteinases, previous experiments demonstrated (43) that bFGF bound to HSPG is protected from proteolysis. Therefore, we next wanted to test whether biologically active endogenous bFGF could be released from the matrix by proteolysis. Two 150-mm dishes of confluent BCE cells were labeled with $^{35}SO_4$ and treated with 5 mg/ml trypsin for 1 min at 37°C. The enzyme was inhibited by addition of soybean trypsin inhibitor (20 mg/ml) and aprotinin (400 KIU/ml). The trypsin releasate was collected, and the few detached cells were removed by centrifugation at 2,000 *g* for 15 min. The released molecules were bound to a 2-ml DE-52 column equilibrated with PBS. The column was washed with 0.25 M NaCl in 0.01 M PO_4 buffer, pH 7.4, and the remaining molecules were eluted with 0.5 M NaCl in the same buffer. Fractions with the highest radioactivity were adjusted to 0.15 M NaCl with water and tested for the presence of bFGF by assaying their ability to stimulate PA production in BCE cells (32, 33, 37). To do this, aliquots of the samples were mixed either with anti-bFGF IgG (200 μg/ml) or with nonimmune rabbit IgG (200 μg/ml), incubated at 37°C for 30 min, and added to BCE cells grown in 24-well Linbro plates. After a 16-h incubation, the cells were extracted, and the PA activity in the samples was measured. As shown in Fig. 4, the endogenous level in the BCE cells was quite low (bar *a*) but could be stimulated 20-fold by the addition of recombinant bFGF (bar *e*). The nonimmune IgG did not inhibit the induction (bar *f*), while the immune IgG was capable of blocking the PA induction in BCE cells observed with 20 μg/ml recombinant bFGF (bar *g*). Control experiments demonstrated that the nonimmune IgG did not affect PA production in the cultures (bar *h*), whereas the immune IgG lowered the basic PA level in the cells. The DE-52 eluate stimulated PA production eightfold (bar *b*). The activity of the eluate was not inhibited by nonimmune IgG, while immune IgG had a clear effect on the expressed PA activity (bars *c* and *d*). These immune IgGs have been shown to be specific for bFGF and not to cross react with other heparin-binding growth factors (22). The above results indicate that proteolytic activity can release bFGF from BCE cells in a form complexed to HSPGs and that this bFGF is biologically active. They also demonstrate that the cells express a bFGF-dependent basic PA activity which is sensitive to the effect of neutralizing anti-bFGF IgG (46).

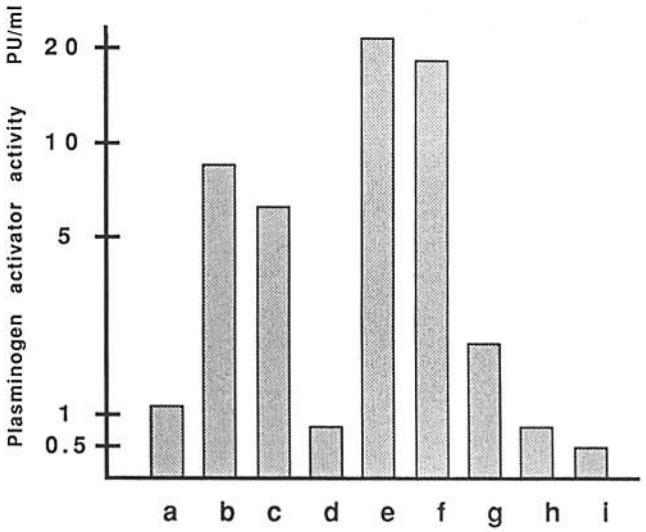

Figure 4. Release by proteolysis of biologically active endogenous bFGF from cultures of BCE cells. Confluent cultures of $^{35}SO_4$-labeled BCE cells were treated with trypsin, DE-52 binding material was isolated, and fractions were tested for PA-stimulating activity as described in the text. PA activity expressed by control cultures (bar *a*) and by cultures with various additions – (bar *b*) DE-52 eluate, (bar *c*) eluate and nonimmune IgG (200 μg/ml), (bar *d*) eluate and anti-bFGF IgG (200 μg/ml), (bar *e*) 20 ng/ml of bFGF, (bar *f*) 20 ng/ml of bFGF and 200 μg/ml of nonimmune IgG, (bar *g*) 20 ng/ml of bFGF and 200 μg/ml of anti-bFGF IgG, (bar *h*) nonimmune IgG (200 μg/ml), (bar *i*) anti-bFGF IgG (200 μg/ml) – is shown.

Effect of bFGF and Transforming Growth Factor β (TGFβ) on the Release of the bFGF-binding Extracellular HSPGs by PA-mediated Proteolysis

Since plasmin was able to cause the release of the bFGF-binding HSPGs from BCE cells, we tested whether the endogenous plasmin activity of BCE cell cultures, mediated through the secretion of PA and activation of plasminogen, could affect the release of extracellular bFGF-binding HSPGs. To test this, BCE cells were treated either with bFGF or with TGFβ. These growth factors have opposite effects on the PA activity expressed by BCE cells (42). While bFGF enhances PA synthesis, treatment of the cells with TGFβ reduces PA synthesis and stimulates PAI-1 synthesis. As demonstrated in Fig. 5, the inclusion of nanogram concentrations of TGFβ-1 in the serum-free growth medium caused a dramatic decrease in the cell-associated PA activity, while the inclusion of bFGF in the culture medium increased PA activity. In the proteolytically active bFGF-treated cultures, the amount of the released metabolically labeled HSPGs was proportional to the plasminogen concentration in the culture medium (Fig. 6, lanes *a–c*). When 4 ng/ml of TGFβ-1 was added to the culture medium during the metabolic labeling of the cells and matrix, the release of HSPGs, mediated by added plasminogen, was substantially inhibited (Fig. 6, lanes *d–f*). Both TGFβ- and bFGF-treated cells contained approximately the same total amount of bFGF-binding HSPGs available for plasmin digestion after 16 h labeling of the cultures, as shown by incubating the cells with plasmin rather than plasminogen (Fig. 6, lanes *g–h*). These results demonstrate that the release of bFGF-binding matrix HSPGs, some of which are probably complexed with the endogenous bFGF, depends upon the concentration of plasmin generated in the cultures which is a reflection of the endogenous pericellular activity.

To further document the role of urokinase as the regulatory enzyme in HSPG release in these experiments, antibodies against bovine urokinase were raised in rabbits. The purified IgG-molecules were used at concentrations of 100, 200, and 500 μg/ml to inhibit the release of $^{35}SO_4$-labeled molecules from cultured BCE cells. Confluent cultures of BCE cells were labeled for 16 h under serum-free conditions, washed thrice, and further incubated for 3 h. The highest concentration of anti-bovine urokinase antibodies almost completely blocked the accumulation of bFGF-binding molecules to the culture medium (Fig. 7). Polyclonal IgG antibodies against human urokinase or against human tPA or preparations of

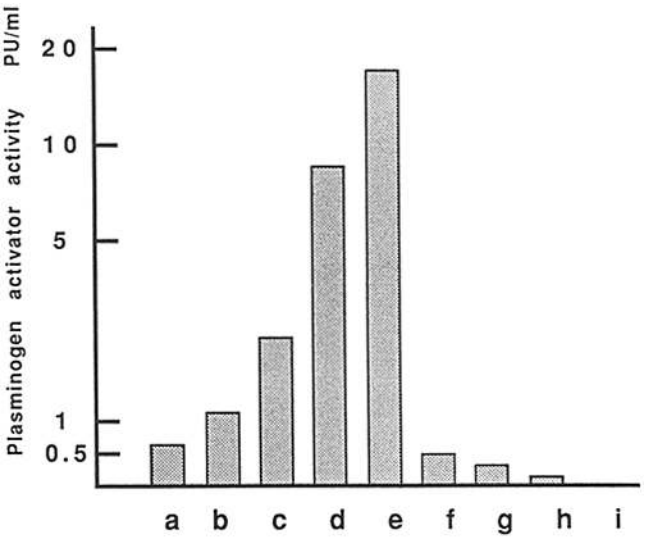

Figure 5. Effect of bFGF and TGFβ on the PA activity in cultures of BCE cells. Increasing amounts of bFGF or TGFβ were added under serum-free conditions to cultures of BCE cells, and PA activity in the cell extracts was determined after a 16-h incubation. (Bars *a–i*) Control cells, 0.3 ng/ml bFGF, 1 ng/ml bFGF, 3 ng/ml bFGF, 10 ng/ml bFGF, 0.1 ng/ml TGFβ, 0.3 ng/ml TGFβ, 1 ng/ml TGFβ, and 3 ng/ml TGFβ, respectively. The data shown in Figs. 4 and 5 are based on quadruplicate PA determinations from two independent experiments. The variation within experimental or control assays was never >20%.

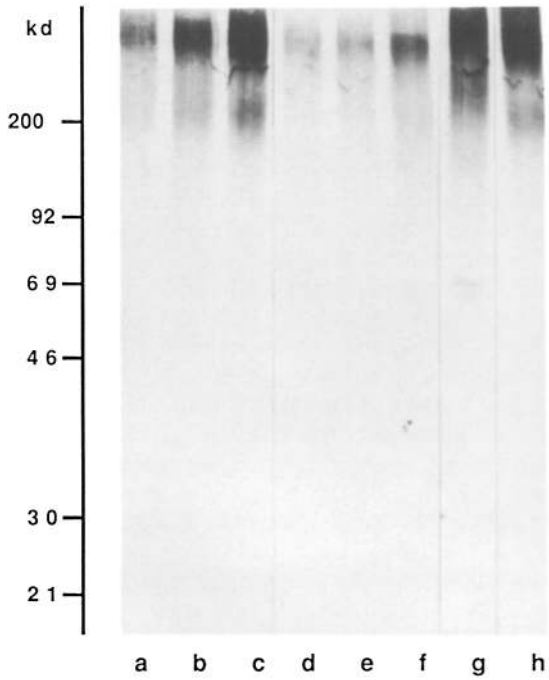

Figure 6. Effect of bFGF and TGFβ on the release of HSPG by plasminogen activation. Cultures of BCE cells were treated either with 10 ng/ml of bFGF or with 4 ng/ml of TGFβ in the presence of $^{35}SO_4$ for 16 h. After the labeling, the cells were washed, increasing amounts of plasminogen were added, and the cultures were incubated for 30 min at 37°C after which aprotinin (200 KIU/ml) was added to inhibit the formed plasmin. Released HSPG molecules were isolated by immobilized bFGF, the samples were analyzed by a 3–16% SDS-PAGE, and the labeled molecules were visualized by autoradiography. (*a–c*) Cultures treated with bFGF; (*d–f*) cultures treated with TGFβ. (*a* and *d*) 1 μg/ml plasminogen; (*b* and *e*) 10 μg/ml plasminogen; (*c* and *f*) 100 μg/ml plasminogen. To demonstrate the total amount of labeled plasmin-sensitive bFGF-binding HSPGs in the cultures, 10 μg/ml of plasmin was added to both bFGF-treated (*g*) and TGFβ-treated (*h*) cells.

nonimmune rabbit IgG at similar concentrations had no detectable effect on the release (data not shown), whereas nonimmune rabbit serum at a 1:20 dilution slightly decreased the accumulation of radiolabeled molecules to the culture medium (Fig. 7).

Combined Effect of TGFβ and bFGF on the Release of HSPG

We have demonstrated in an earlier study that pretreatment of BCE cells with TGFβ decreases or completely prevents the rapid stimulation of PA activity by bFGF (42). Since the release of HSPG appears to be closely dependent on the PA activity expressed by BCE cells, we wanted to examine whether a similar kind of inhibitory effect by TGFβ pretreatment could be detected on the release of HSPG from BCE cells. Incubation of the cells with TGFβ for 6 h before the addition of bFGF decreased the amount of released HSPG compared with release from cultures incubated in medium alone or with bFGF (Fig. 8). The release was close to that seen in cultures treated with TGFβ alone.

Discussion

bFGF is synthesized by endothelial cells in culture (32, 48, 52) and has been shown to affect several cellular properties in an autocrine fashion (46). The growth factor binds readily to extracellular matrix structures and can be recovered from preparations of isolated matrix from cultures of endothelial cells (29, 52). We have previously shown that soluble heparan sulfate molecules synthesized by endothelial cells bind bFGF and protect it from proteolytic inactivation (43). In the present article, we show that the bFGF-binding HSPGs are located in the extracellular structures of BCE cells and suggest a mechanism for the release of these bFGF-binding molecules and bFGF by the physiologically relevant protease, plasmin. The release of extracellular HSPGs can be modulated by factors that affect the proteolytic balance in the pericellular environment. This provides a mechanism for controlling bFGF release from the matrix and/or cell surface.

The BCE cells seem to have two principal bFGF-binding HSPGs, which are located in separate compartments of the cell. The larger 800-kD molecule remains attached to the dish after detergent extraction of the cultures and seems to be a component of the extracellular matrix. The smaller 250-kD molecule is readily dissolved by nonionic detergents and thus may represent a membrane proteoglycan as described in some other cell culture models (20, 23).

The extracellular matrix is considered to have an important function in guiding and regulating cell morphology, growth, and differentiation in vivo. At sites of active tissue remodeling and growth, a primary and often dominant feature is hydrolysis of matrix components via secretion of lytic

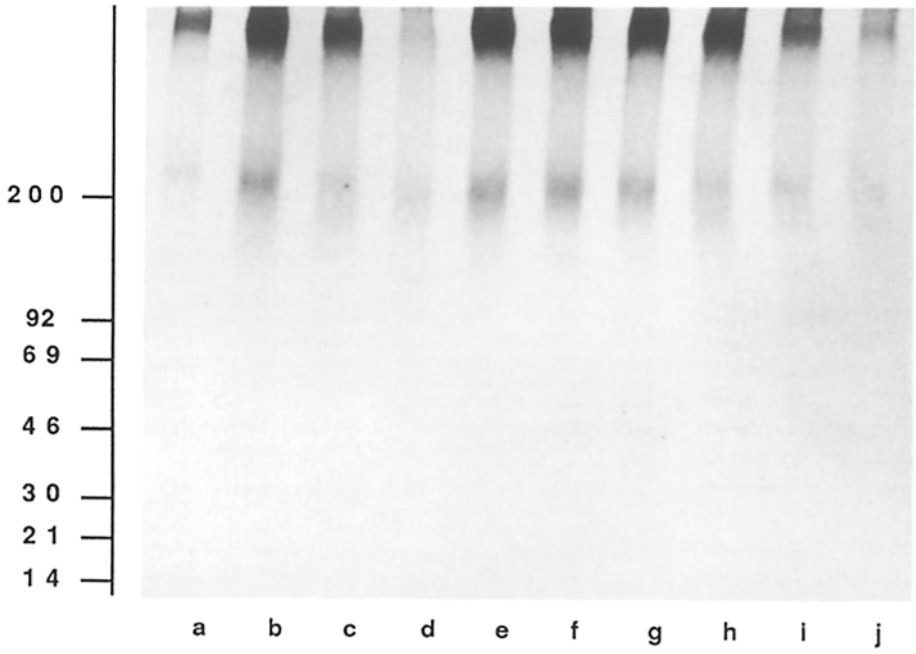

Figure 7. Effect of neutralizing anti-bovine urokinase antibodies on the release of $^{35}SO_4$-labeled bFGF-binding HSPG released by BCE cells. The cells were labeled for 16 h, washed, and incubated in the presence of 10 μg/ml plasminogen (*a–j*) and 200 KIU/ml aprotinin (*a*); plasminogen alone (*b*); 5% nonimmune rabbit serum (*c*); 5% immune anti-bovine urokinase antiserum (*d*); 50, 200, and 500 μg/ml nonimmune rabbit IgG (*e–g*, respectively); or 50, 200, and 500 μg/ml anti-bovine urokinase IgG (*h–j*, respectively). After the incubation, aprotinin was added, the released molecules were isolated by protein A–immobilized bFGF, the samples were analyzed by a 3–16% SDS-PAGE, and the labeled molecules were visualized by autoradiography.

enzymes by the proliferating cells (31). This may well lead to the release of biologically active fragments of the degraded matrix components and to the release of matrix-bound growth factors. The soluble heparan sulfate–bFGF complex is biologically active and does not bind to the low affinity matrix-binding sites as does the uncomplexed bFGF (43). Thus, complex formation with heparan sulfate molecules would facilitate the diffusion of the growth factor to more distant sites. For example, release of bFGF from injured corneal epithelial basement membrane has been suggested to have a role in corneal neovascularization (12). The basement membrane is rich in heparan sulfates, and heparinase can release bFGF-related activity from isolated corneal membranes (12). However, a prominent feature of sterile chronic corneal ulcers is an increased plasmin activity detectable in the corneal fluid (44). Thus, a possible mechanism is the release of heparan sulfate–bFGF complexes by proteolysis rather than bFGF alone from the injured area. The complexed, biologically active bFGF is resistant to inactivation in the proteinase-rich milieu and could freely diffuse to the vascularized area to initiate corneal angiogenesis.

PAs are serine proteinases found in several tissues in vivo and are synthesized by many cell types in vitro (6). We propose that the activation of the abundant proenzyme plas-

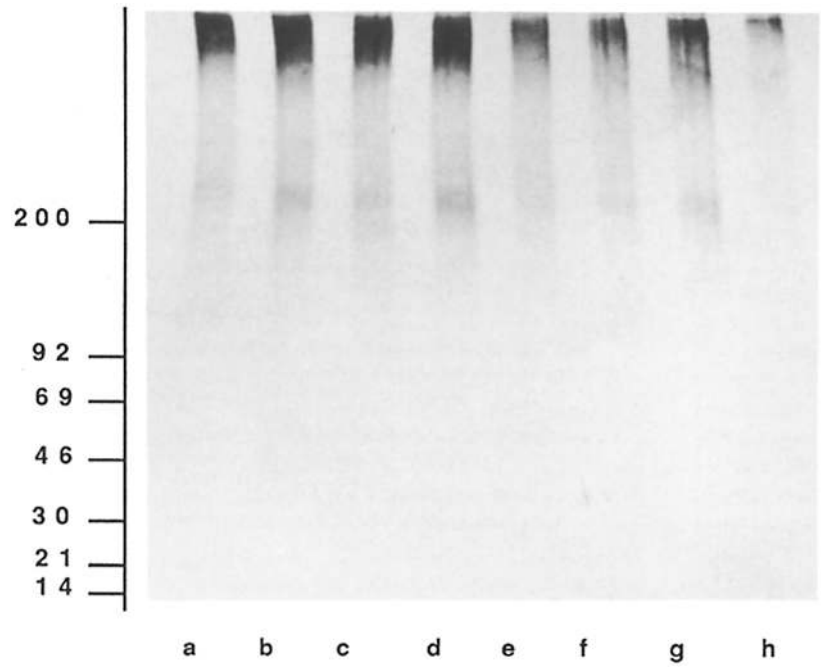

Figure 8. Release of $^{35}SO_4$-labeled bFGF-binding HSPG after combined treatment with TGFβ and bFGF. Cultures of BCE cells were pretreated either with 10 ng/ml of bFGF (*a* and *b*) or with 4 ng/ml of TGFβ (*e* and *f*) or incubated in serum-free medium (*c*, *d*, *g*, and *h*). After 6 h, new medium containing the label, 10 μg/ml of plasminogen, and bFGF or TGFβ was added to the cultures (3 ng/ml of bFGF in *a*, *c*, and *e*; 10 ng/ml of bFGF in *b*, *d*, and *f*; 1 ng/ml of TGFβ in *g*; 4 ng/ml of TGFβ in *h*). The cultures were further incubated for 16 h, and the media were collected. Aprotinin was added, the released molecules were isolated by protein A–immobilized bFGF, samples were analyzed by a 3–16% SDS-PAGE, and the labeled molecules were visualized by autoradiography. Release of bFGF-binding HSPG after bFGF stimulation (*a–f*) is less in the TGFβ-pretreated cultures (*e* and *f*) than in cultures preincubated in medium alone (*c* and *d*) or with bFGF (*a* and *b*) and is close to the release of HSPG in cultures treated with TGFβ alone (*g* and *h*).

minogen mediates the release of HSPG from extracellular structures and thereby promotes the release of bFGF in a biologically active and soluble form. To demonstrate the effect of the level of expressed PA activity on the release of extracellular HSPGs, we treated the cells either with bFGF to enhance PA level or with TGFβ to suppress it (42). Treatment of BCE cells with TGFβ resulted in greatly diminished formation of plasmin and decreased degradation of the matrix heparan sulfate proteoglycans. Addition of purified IgG-antibodies against bovine urokinase in the incubation medium during collection of the metabolically labeled bFGF-binding HSPGs almost completely blocked the release of these molecules from the cell layer. It is interesting to note that pretreatment of cultures with TGFβ blocks the stimulation of the cells by bFGF (42). Whether this interaction could partly result from decreased proteolytic activity caused by TGFβ treatment and subsequently from decreased availability of the matrix-bound bFGF to the high affinity receptor (30) has not been documented. However, we have now demonstrated decreased release of HSPG from BCE cells after bFGF stimulation when the cells have been pretreated for 6 h with TGFβ.

A role for proteolytic activity in cellular proliferation is suggested by an earlier publication by Bergman et al. (4). Addition of a proteinase inhibitor, protease nexin I, to cultures of fibrosarcoma cells resulted in a decreased degradation of isolated endothelial cell matrix by the fibrosarcoma cells plated on top of the matrix. Addition of protease nexin 1 also caused a decrease in the proliferation rate of the fibrosarcoma cells. A possible explanation for this observation is that a decrease in matrix degradation results in decreased release of growth-promoting peptides from the cell matrix. Growth factors, which induce PAI-1, such as TGFβ, are known to act as effective growth inhibitors in several experimental models (34). Part of their action may well be stabilization of the matrix structures via increased accumulation of proteinase inhibitors, such as PAI-1 (26) or the tissue inhibitor of metalloproteinases (9), into the extracellular space. The growth substratum in endothelial cells contains functionally active, stabilized PAI-1 (27, 28). This would result in decreased release of matrix-bound growth factors.

The plasminogen–plasmin system works predominantly at cell-to-cell or cell-to-substrate contact areas (41). A close contact is required in many experimental models, where the differentiation or proliferation of one cell type is mediated by the presence of another cell. Such an interaction can clearly be demonstrated in the bone marrow, where differentiation of blood cells requires a cell-to-cell contact with the local stromal fibroblasts (8, 13). Colony-stimulating factors necessary for differentiation of the stem cells are suggested to bind to stromal cell matrix (14), and isolated heparan sulfate has been suggested to bind growth factors necessary for the stem cell proliferation (39). Thus, the degradation of the HSPG core protein by plasmin may be part of a more general mechanism by which growth-regulating factors are made available to cellular receptors. Regulation of the pericellular proteolytic activity could be of primary importance in this process.

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