Heparin and Heparan Sulfate Increase the Radius of Diffusion and Action of Basic Fibroblast Growth Factor

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Abstract. The radius of diffusion of basic FGF (bFGF) in the presence and in the absence of the glycosaminoglycans heparin and heparan sulfate was measured. Iodinated ^{125}I-bFGF diffuses further in agarose, fibrin, and on a monolayer of bovine aortic endothelial (BAE) cells in the presence of heparin than in its absence. Heparan sulfates affected the diffusion of ^{125}I-bFGF in a manner similar to, though less pronounced than, heparin. When applied at the center of a monolayer of BAE cells, bFGF plus heparin stimu-
lated morphological changes at a 10-fold greater radius than bFGF alone. These results suggest that bFGF-heparin and/or heparan sulfate complexes may be more effective than bFGF alone in stimulating cells located away from the bFGF source because the bFGF-glycosaminoglycan complex partitions into the soluble phase rather than binding to insoluble glycosaminoglycans in the extracellular matrix. Thus, the complex of bFGF and glycosaminoglycan may represent one of the active forms of bFGF in vivo.

BASIC FGF (bFGF)[1] is found in essentially all normal tissues (Joseph-Silversten and Rifkin, 1987; Rifkin and Moscatelli, 1989; Burgess and Maciag, 1989), is probably present in all vertebrates, and is highly conserved among different species (Gospodarowicz et al., 1986a,b; Rifkin and Moscatelli, 1989). bFGF induces cellular proliferation, stimulates protease secretion and chemotaxis, delays senescence, and affects protein synthesis and hormone release in a variety of mesoderm and neuroectoderm-derived cells (Gospodarowicz et al., 1986a, b; Burgess and Maciag, 1989; Rifkin and Moscatelli, 1989). This growth factor has been detected in the majority of tumor cell lines tested (Moscatelli et al., 1986), and has been implicated as a contributing factor in the neovascularization of tumors (Folkman et al., 1971; Folkman et al., 1988). However, bFGF, which is a highly positively charged molecule at physiologic pH (pI = 9.8) (Lobb et al., 1986a), binds avidly to negatively charged proteoglycans and appears not to be freely diffusible in its extracellular microenvironment (Vlodavsky et al., 1987b). Indeed, it is not found in significant amounts in serum or in medium conditioned by cells which produce it (Gauthier et al., 1987; Vlodavsky 1987a,b).

One of the interactions between bFGF and negatively charged molecules that has been studied and proposed to be of biological significance is the interaction between bFGF and heparin (Klagsburn and Shing, 1985; Gospodarowicz and Cheng, 1986; Saksela et al., 1988; Sommer and Rifkin, 1989; Saksela and Rifkin, 1990; Uhlrich et al., 1986). bFGF was initially purified using heparin-Sepharose chromatogra-

phy (Gospodarowicz et al., 1984; Shing et al., 1984) and considerable speculation has been made concerning the potential significance of the interaction between bFGF and heparin-like species in the extracellular matrix (ECM) during angiogenesis and tumor growth (Folkman et al., 1988; Baird and Ling, 1987; Saksela et al., 1988; Flaumenhaft et al., 1989; Presta et al., 1989). bFGF has been isolated from ECM produced in vitro (Vlodavsky et al., 1987a) and from basement membranes synthesized in vivo (Folkman et al., 1988) and has been demonstrated to interact specifically with heparan sulfate proteoglycans in both matrices. Proteolytic degradation of the ECM by plasmin releases an active form of the bFGF-heparan sulfate complex (Saksela and Rifkin, 1990), and bFGF complexed in this manner is protected from proteolytic degradation (Sommer and Rifkin, 1989; Saksela et al., 1988).

In addition to rendering bFGF resistant to proteolytic degradation, the interaction with soluble heparan sulfate might also prevent bFGF from binding to immobilized heparan sulfate proteoglycans in the ECM. Since bFGF in a bFGF-heparan sulfate complex would have its heparin-binding site(s) unavailable to bind to insoluble heparan sulfate proteoglycans in the ECM, the bFGF would tend to partition into the soluble phase and diffuse freely in an environment rich in immobilized glycosaminoglycans. In contrast, uncomplexed bFGF would be bound by such matrix molecules and be unable to diffuse. Thus, despite the fact that the bFGF-heparan sulfate complex would be larger than the bFGF molecule alone, the complex would diffuse further than free bFGF when released from a discrete source and demonstrate an increased radius of action. Since bFGF binds avidly to immobilized heparan sulfate proteoglycans in vivo

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1. *Abbreviations used in this paper*: BAE, bovine aortic endothelial; BCE, bovine capillary endothelial; bFGF, basic FGF; ECM, extracellular matrix.

(Folkman et al., 1988), it is possible that the active species responsible for stimulating target cells in vivo is a bFGF-heparan sulfate complex.

In this paper, we have examined the partition properties of free bFGF and bFGF plus soluble glycosaminoglycans under several conditions. We have found that the bFGF-heparin complex diffuses further than bFGF alone in agarose, in fibrin, and on a cell monolayer, all of which have immobilized binding sites for bFGF. The bFGF-heparin complex also stimulates morphological changes in a significantly larger area than bFGF alone when released from a defined source on a cellular monolayer. Heparan sulfate affects the partition of bFGF in a manner similar to that of heparin. We propose that these results derive from the greater partition of the bFGF-heparin or heparan sulfate complex into the aqueous mobile phase rather than binding to the insoluble matrix.

Materials and Methods

Reagents

Porcine intestinal mucosa heparin and bovine kidney heparan sulfate were obtained from Sigma Chemical Co. (St. Louis, MO). Equine heart cytochrome c was purchased from Calbiochem-Behring Corp. (San Diego, CA). Fibrinogen was obtained from Miles Scientific Division (Naperville, IL), and thrombin was obtained from Sigma Chemical Co. Human placental bFGF was purified as described previously (Moscatelli et al., 1986; Presta et al., 1986). Recombinant human bFGF was a gift from Synergen Inc. (Boulder, CO).

Cells

Bovine capillary endothelial (BCE) and bovine aortic endothelial (BAE) cells were isolated as described previously (Folkman et al., 1979) and grown at 37°C in alpha modified Eagle's medium (a-MEM) (Flow Laboratories, Inc., McLean, VA) supplemented with 5% calf serum (Flow Laboratories Inc.). Serum-free conditioned medium was collected from cultured BCE cells every 4 d and stored at −70°C.

Labeling of bFGF

bFGF was labeled with ^{125}I (17 Ci/mg) (New England Nuclear, Boston, MA) using Iodo-Gen (Pierce Chemical Co., Rockford, IL) as previously described (Neufeld and Gospodarowicz, 1985; Moscatelli, 1987).

Diffusion in Agarose Gels

To assay the diffusion of cytochrome c in an agarose gel, 6 ml of a hot 0.8% agarose (Seakem HGT agarose; FMC Corp., Rockland, ME) solution in water were transferred to a 60-mm tissue culture dish and allowed to gel at room temperature on a leveling plate. A well was then made in the center of the gel using a 5-mm-diam core punch. 15 μl of a 100 mg/ml solution of cytochrome c plus 15 μl of either distilled water, heparin (100 mg/ml), suramin (1 mg/ml), or protamine sulfate (60 mg/ml) were added to the center well. The culture dishes were maintained on a leveling plate in a humid environment for 48 h at 37°C and then photographed.

To assay the diffusion of ^{125}I-bFGF in an agarose gel, 2 ml of a 0.8% agarose solution were transferred to a 35-mm tissue culture dish and allowed to gel. A 5-mm well was punched in the center of the gel and 15 μl of a 10 ng/ml solution of ^{125}I-bFGF plus 15 μl of either distilled water, heparin (100 mg/ml), suramin (1 mg/ml), or protamine sulfate (60 mg/ml) were added to the well. After a 48-h incubation in a humid environment at 37°C, the agarose gel was dried under a heat lamp and the side of the culture dish was removed using a soldering iron. The bottom of the culture plate containing the dried film of agarose was exposed to autoradiography Kodak film for the indicated period of time and developed. The radioactivity in the dried film was quantitated by transferring 0.5 ml of 0.5% Triton X-100 onto the culture plate and scraping the film into a test tube with a spatula. The radioactivity associated with the film was measured in a Multi-Prias I

gamma scintillation counter (Packard Instrument Co., Inc., Downers Grove, IL).

For experiments analyzing the diffusion of ^{125}I-bFGF in the presence of commercial heparan sulfate, autoradiograms were analyzed by scanning across four different diameters of the autoradiographic image using laser densitometry. The amount of radioactivity at the source of ^{125}I-bFGF was quantitated by measuring the peak height of the densitometer tracing. The peak height is defined as the distance between the densitometer measurement of grain density at background and the measurement of grain density at the source of ^{125}I-bFGF. The relative area of ^{125}I-bFGF diffusion was calculated by measuring the peak width of the densitometer tracings and using this measurement to represent the diameter of diffusion of ^{125}I-bFGF.

Diffusion of bFGF in Fibrin Gels

To assay the diffusion of ^{125}I-bFGF in a fibrin gel, 2 ml of fibrinogen (3 mg/ml) in PBS were transferred to a 35-mm tissue culture dish. 10 μl of thrombin (100 U/ml) were mixed into the fibrinogen solution to initiate polymerization, and the culture dish was placed on a leveling plate. A 12-mm-diam Millicell-HA filter with legs (Millipore Co., Bedford, MA) from which the filter had been removed was placed in the center of the culture dish to form a well and the fibrinogen was allowed to polymerize for 1 h at 22°C. 250 μl of ^{125}I-bFGF (0.1 ng/ml) were transferred to the Millicell with or without heparin (10 ng/ml). After a 24-h incubation, the Millicell was removed, and the gel was dried under a heatlamp. The side of the culture dish was removed and the bottom of the culture plate was placed on autoradiography film. The film was exposed for the indicated time and developed. The developed film was analyzed by scanning laser densitometry to determine the distance of ^{125}I-bFGF diffusion in the fibrin gel. The extent of diffusion was calculated by subtracting the radius of the Millicell from which the ^{125}I-bFGF originated (5 mm) from the radius of the area through which ^{125}I-bFGF diffused after a 24-h incubation.

### Diffusion of bFGF on a Cellular Monolayer

Confluent cultures of BAE cells in 60-mm tissue culture dishes were washed twice with PBS and 2 ml of serum-free a-MEM was added. A 12-mm Millicell, from which the legs had been removed, was gently placed on the center of the monolayer. 200 μl of a-MEM containing 15 ng of ^{125}I-bFGF was added alone or with heparin (100 μg/ml) to the Millicell and diffusion was allowed to take place without perturbation for the indicated time at 37°C on a leveling plate. After incubation, the Millicell and the 2 ml of medium were quickly removed. The cells were washed twice with PBS, fixed with methanol, and stained with Wright-Giemsa stain (J. T. Baker Chemical Co., Phillipsburg, NJ). The outside bottom surface of the culture dish was scored with concentric circles using a syringe needle secured to a compass to delineate regions of increasing distance away from the area where the Millicell has been placed. Photographs were taken of each region of the culture dish using an Olympus C-35AD 33-mm camera attached to a Diavert light microscope (E. Lietz, Inc., Rockleigh, NJ). The side of the culture dish was removed and the bottom of the dish placed on autoradiographic film, exposed for the indicated time, and developed. The radioactivity associated with the cellular monolayer was quantitated by transferring 1 ml of 0.5% Triton-X 100 onto the culture plate and scraping the dried monolayer into a test tube with a spatula.

Purification of Heparan Sulfates Secreted by BCE Cells

Triton X-100 was added to 250 ml of conditioned medium from cultured BCE cells to a final concentration of 0.5% and the conditioned medium was chromatographed on 10 ml of DE 52 (Whatman Inc., Clifton, NJ) in a 2 × 15 cm column equilibrated with 0.01 M PO_4, pH 7.4, 0.15 M NaCl, and 0.5% TX-100, according to the procedure of Saksela et al. (1988). The column was washed with equilibration buffer followed by 0.25 M NaCl in the same buffer. The remaining bound material was eluted with 0.5 M NaCl, resulting in a relatively selective release of the glycosaminoglycans and proteoglycans (Yanagishita et al., 1987). The eluate was diluted to 0.25 M NaCl, and applied to 1 ml of DE 52. The column was washed with 0.01 M Tris-HCl, pH 7.4, containing 0.25 M NaCl and 0.5% TX-100. The bound material was eluted with 2.0 M NaCl in the above buffer. The fractions were analyzed for glycosaminoglycan and proteoglycan concentration by the method of Bitter and Muir (1962) and were assayed for their ability to increase the diffusion of ^{125}I-bFGF in agarose. Diffusion was assessed by au-

Cytochrome C Diffusion in Agarose

cyto C+Protamine   cyto C+Suramin

cyto C+Heparin   cyto C   Agarose
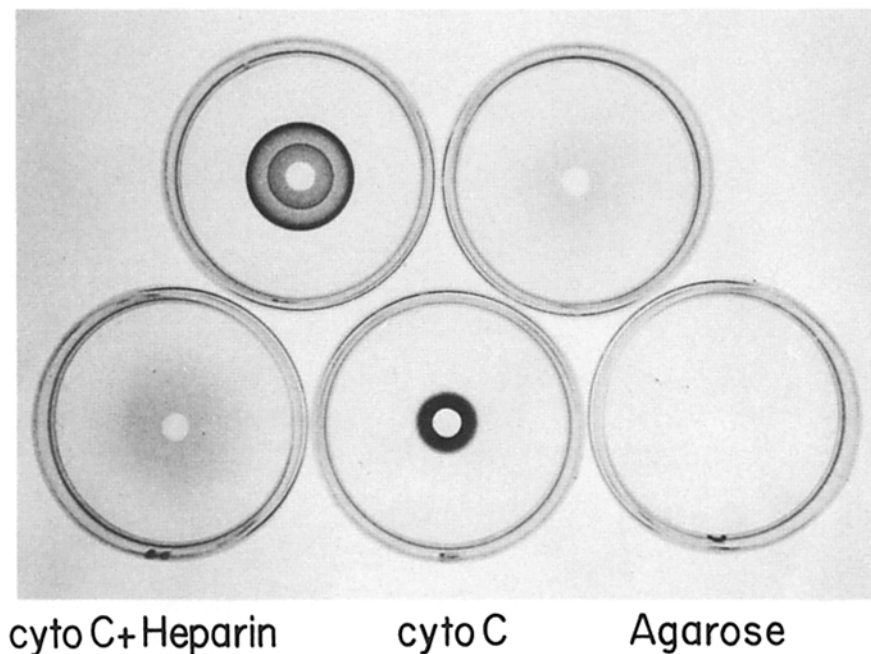
Figure 1. Diffusion of cytochrome c in agarose gels in the absence or presence of heparin, suramin, or protamine sulfate. 15 µl of cytochrome c (100 mg/ml) were transferred to a well in the center of an agarose gel. 15 µl of distilled water, heparin (100 mg/ml), suramin (1 mg/ml), or protamine sulfate (60 mg/ml) were then mixed with the cytochrome c solution. The gels were photographed after a 48-h incubation when no further diffusion of cytochrome c could be observed.

toradiography as described above and the autoradiograms were analyzed by scanning laser densitometry.

Results

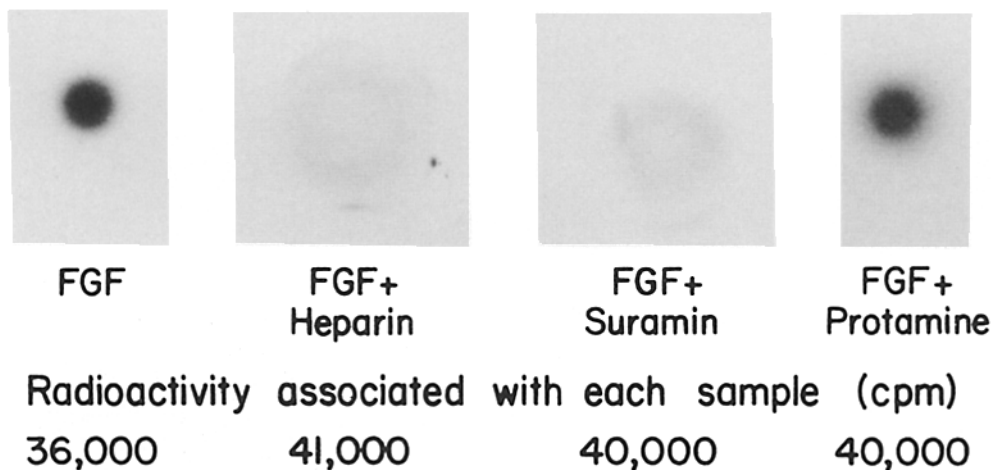### Diffusion of Cytochrome c in Agarose Gels

Initial experiments were designed to determine whether the radius of diffusion of a positively charged polypeptide through a negatively charged matrix would be decreased by interactions between the polypeptide and the immobilized negative charges. If a decrease in the radius of diffusion did occur, then we could determine whether this effect could be overcome by complexing the polypeptide with soluble negatively charged molecules such as heparin and suramin. We used cytochrome c because (*a*) like bFGF, it has a basic pI; (*b*) its molecular weight (12 kD) is comparable to that of bFGF (18 kD); and (*c*) its diffusion can easily be observed owing to its characteristic red color. Agarose was used because it is known to contain immobilized sulfate groups.

Cytochrome c diffuses poorly in agarose as determined by the dark red ring tightly encircling the well from which it originates (Fig. 1). In contrast, cytochrome c diffuses significantly further in the presence of heparin (Fig. 1). A second sulfated molecule which has been shown to interact with bFGF is suramin (Sato and Rifkin, 1988). Suramin binds to bFGF and blocks its interaction with matrix as well as with its receptor. When the diffusion of a mixture of cytochrome

c and suramin was assayed, the cytochrome c diffused nearly to the edge of the culture dish. These results indicate that the positively charged cytochrome c complexes with the negatively charged sulfate moieties of heparin and suramin forming soluble complexes which can diffuse within an environment of immobilized negative charges. In contrast, cytochrome c in the absence of a polyanion is bound to the insoluble matrix and is unable to diffuse.

Fig. 1 also demonstrates that the highly basic molecule protamine sulfate can increase the diffusion of cytochrome c by a mechanism distinct from that attained by the addition of heparin or suramin. With the addition of protamine sulfate, the cytochrome c moved a defined distance from the central source and did not diffuse past that point. A reason for this may be that protamine binds to the sulfate moieties in the agarose thereby reducing the number of anionic sites available for interaction with the cytochrome c in the immediate vicinity of the central well. This permits the cytochrome c to diffuse further into the agarose until it reaches a point at which all of the protamine sulfate has been bound to the agarose. The cytochrome c then ceases to diffuse further. This hypothesis is supported by an experiment in which additional protamine was added to the well when the diffusion of the cytochrome c had ceased. Under these conditions, the cytochrome c diffused further and again stopped, creating a new border with a larger radius than the first (data not shown). This probably resulted from the neutralization of a greater area of agarose by the additional protamine.

^{125}I-bFGF diffusion in agarose

| FGF | FGF+ Heparin | FGF+ Suramin | FGF+ Protamine |

Radioactivity associated with each sample (cpm)

| 36,000 | 41,000 | 40,000 | 40,000 |

Figure 2. Diffusion of ^{125}I-bFGF in agarose gels in the absence or presence of heparin, suramin, or protamine sulfate. 15 μl ^{125}I-bFGF (10 ng/ml) were transferred to a well in the center of an agarose gel. 15 μl of distilled water, heparin (100 mg/ml), suramin (1 mg/ml), or protamine sulfate (60 mg/ml) were then mixed with the ^{125}I-bFGF solution. After a 48-h incubation, the gels were dried under a heat lamp, the side of the culture dishes in which they were contained was removed, and autoradiography was performed. The film was developed after a 10-h exposure. The total amount of radioactivity in each of the dishes was roughly equivalent: ^{125}I-bFGF alone (36,000 cpm), with heparin (41,000 cpm), with suramin (40,000 cpm), and with protamine sulfate (40,000 cpm).

Diffusion of ^{125}I-bFGF in Agarose Gels

The diffusion of bFGF in agarose was visualized by using ^{125}I-bFGF in experiments similar to those performed with cytochrome c, drying the agarose, and exposing the dry agarose to autoradiographic film. The results indicate that the same factors which affect the radius of diffusion of cytochrome c in agarose also affect the radius of diffusion of bFGF in agarose (Fig. 2). By itself, ^{125}I-bFGF diffused 3.5 mm into the agarose gel from the edge of the well from which it originates over 48 h. Both heparin and suramin markedly increased the radius of diffusion, enabling the ^{125}I-bFGF to diffuse 9.5 and 8.0 mm, respectively, into the gel. Thus, ^{125}I-bFGF diffused ~2.7-fold further in the presence of heparin and 2.3-fold further in the presence of suramin than in the absence of these molecules. The decreased density of grains in the autoradiograms of the heparin and suramin

samples is due to dilution of ^{125}I-bFGF over a larger surface as the total amount of radioactivity in each dish was similar. As was seen with cytochrome c, protamine had a less dramatic effect in promoting diffusion. In the presence of protamine, ^{125}I-bFGF diffused 5.0 mm into the agarose gel: 1.4-fold further than in the absence of protamine. These results support the conclusions that heparin can bind positively charged regions of both cytochrome c and bFGF and neutralize interactions with immobilized negative charges in the matrix.

### Diffusion of ^{125}I-bFGF in Fibrin Gels

Several reports have demonstrated the ability of fibrin to support in vitro angiogenesis (Montesano et al., 1985; Nicosia et al., 1983). For this reason, we analyzed the diffusion of ^{125}I-bFGF in fibrin. Fibrinogen has an isoelectric point of

Diffusion of ^{125}I-bFGF in fibrin

| ^{125}I-bFGF | ^{125}I-bFGF-heparin |
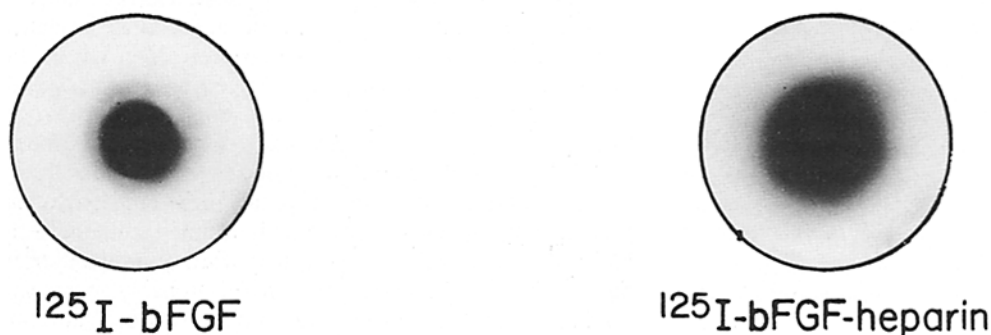
Figure 3. Diffusion of ^{125}I-bFGF in fibrin gels in the absence or presence of heparin. A Millicell well from which the filter had been removed was placed in the center of a 35-mm dish (represented by the outer circle of each figure) containing fibrinogen. A gel was formed by the addition of thrombin and ^{125}I-bFGF was transferred to the Millicell well in the absence or presence of heparin. Diffusion was assayed as described in Fig. 2 after a 24-h incubation. The film was developed after a 14-h exposure.

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^{125}I-bFGF diffusion
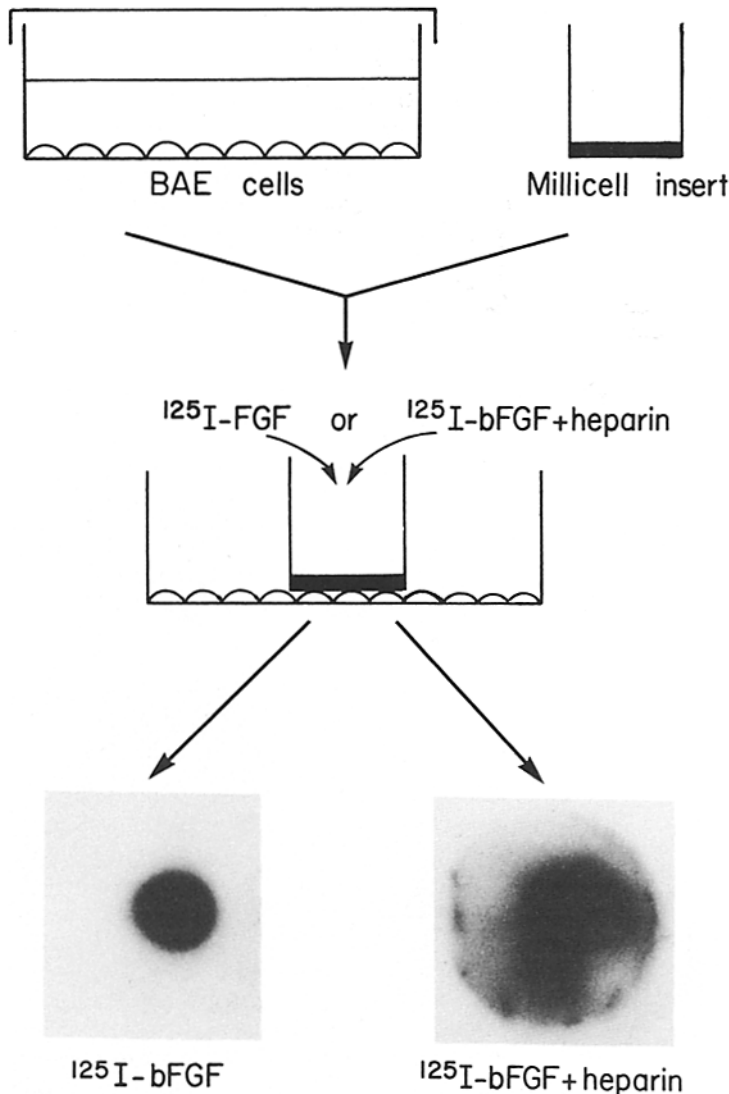
Figure 4. Diffusion of ^{125}I-bFGF on a cellular monolayer in the absence or presence of heparin. A legless Millicell was placed in the center of a confluent monolayer of BAE cells. 200 μl of ^{125}I-bFGF (15 ng/ml) with or without heparin (100 μg/ml) were transferred to the Millicell. After a 24-h incubation the cells were fixed, the sides of the culture dish were removed, and autoradiography was performed. The film was developed after a 96-h exposure.
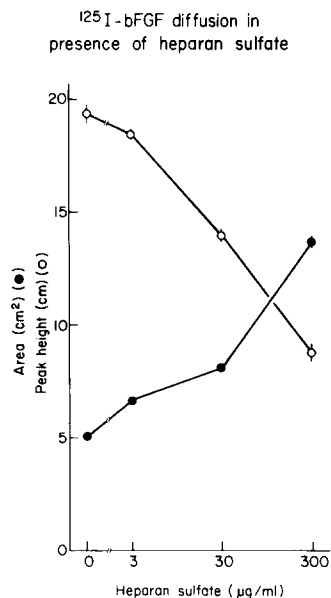
5.5 and should interact with bFGF. Fig. 3 demonstrates that the diffusion of ^{125}I-bFGF is restricted in a fibrin gel. Heparin, however, increased its diffusion. Scanning laser densitometry showed that ^{125}I-bFGF alone diffused 2.5 mm from the edge of the Millicell during a 24-h incubation, while ^{125}I-bFGF complexed with heparin diffused 6.0 mm from the edge of the Millicell. Thus, ^{125}I-bFGF diffused in fibrin approximately 2.4-fold further in the presence of heparin than in its absence.

Diffusion of bFGF on a Cellular Monolayer

Since heparin increased the radius of diffusion of bFGF in agarose, we attempted to determine whether heparin increased the radius of diffusion of bFGF on a cellular monolayer. It seemed possible that bFGF released onto a small area of the monolayer in the absence of heparin would be bound by immobilized (insoluble) heparan sulfate proteoglycan in the matrix in the immediate vicinity of its re-

lease. In contrast, bFGF released onto a small area in the presence of heparin would not be bound by heparan sulfate proteoglycans. It would diffuse further away from the site of release, being bound primarily by bFGF receptors, which are 10-fold less abundant within the monolayer than heparan sulfate proteoglycan binding sites (Moscatelli, 1987).

Fig. 4 shows that bFGF originating from a Millicell placed in the center of a monolayer diffused further in the presence of heparin than in its absence. The radius of diffusion in the presence of heparin was difficult to quantitate because of the asymmetric pattern of diffusion. However, the result is marked and reproducible. The association of uncomplexed ^{125}I-bFGF with the monolayer probably represents binding to both bFGF-receptors and heparan sulfate proteoglycans immediately under and surrounding the source of ^{125}I-bFGF. The radioactivity associated with the monolayer in the presence of ^{125}I-bFGF and heparin probably represents binding to bFGF-receptors since soluble heparin at the con-

¹²⁵I-bFGF diffusion in presence of heparan sulfate

Figure 5. Diffusion of ¹²⁵I-bFGF in agarose gels in the presence of increasing concentrations of commercial bovine kidney heparan sulfate. Diffusion experiments as described in Fig. 2 were performed using ¹²⁵I-bFGF in the absence or presence of increasing amounts of heparan sulfate. The radius of diffusion and density of autoradiographic grains at the source of ¹²⁵I-bFGF were determined by scanning densitometry. The autoradiograms were scanned across four different diameters and the results averaged. Open circles and vertical bars represent the means and standard deviations of the measurements of the peak height of an autoradiographic tracing. Closed circles and vertical bars represent the means and standard deviations of an area representing the diffusion of ¹²⁵I-bFGF determined as described in Materials and Methods.

centration used in this experiment (10 μg/ml) is known to prevent binding of bFGF to heparan sulfate proteoglycans in the ECM (Moscatelli, 1987; Flaumenhaft et al., 1989). The reason for the symmetric pattern of ¹²⁵I-bFGF in the heparin containing samples is unclear but may relate to differences in the composition of the cell monolayer or convection currents in the culture dish.

To demonstrate that the differences in the radius of diffusion between the two conditions did not result from an increased ability of ¹²⁵I-bFGF to be released from the Millicell in the presence of heparin, we measured the amount of radioactivity remaining in the Millicell after the 24-h incubation period. Such measurements suggested that ∼20% of the total ¹²⁵I-bFGF escaped from the Millicell over the 24-h incubation period in the absence or presence of heparin. The difference in diffusion is also not the result of increased binding of the bFGF-heparin complex to the ECM of the BAE cells compared to the binding to bFGF alone since ∼8% of the total ¹²⁵I-bFGF applied to the Millicell was associated with the monolayer in the absence or presence of heparin after a 24-h incubation. Thus, we conclude that the increased radius of diffusion of ¹²⁵I-bFGF on a cellular monolayer in the presence of heparin results from the ability of heparin to prevent the binding of ¹²⁵I-bFGF to immobilized heparan sulfate proteoglycans in the ECM and thereby allow the ¹²⁵I-bFGF-heparin complex to diffuse freely on the monolayer.

Heparan Sulfates Increase the Radius of Diffusion of bFGF

In vivo, bFGF is more likely to encounter heparan sulfates than heparin. For this reason, the ability of both commercial heparan sulfates and heparan sulfates purified from BCE cells to increase the radius of diffusion of ¹²⁵I-bFGF in agarose was investigated. Fig. 5 shows that commercial

heparan sulfate increased the radius of diffusion of bFGF in agarose in a dose-dependent manner. The measurements of peak height and relative area demonstrate that the density of radioactivity at the ¹²⁵I-bFGF source decreases as the area of diffusion increases. Heparan sulfate also increased the diffusion of ¹²⁵I-bFGF on a cellular monolayer (data not shown). The dose of heparan sulfate necessary to yield an increased radius of diffusion is ∼100-fold greater than the doses of heparin necessary to achieve a similar increase (data not shown). As shown in Table I, glycosaminoglycans and proteoglycans purified from BCE cells increased the diffusion of bFGF agarose. Presumably, heparan sulfate acts as heparin does: by binding to the residues of the bFGF molecule that interact with sulfate moieties in the agarose. However, the heparan sulfate is less effective than heparin probably because of its lower degree of sulfation.

## Increased Radius of Cellular Stimulation with bFGF-Heparin

One consequence of bFGF stimulation is a characteristic change in cell morphology. Upon exposure, cells become elongated and develop long, thin processes. Fig. 6 demonstrates that the ¹²⁵I-bFGF-heparin complex stimulates morphological change at a greater radius from its source than bFGF alone. In these experiments, ¹²⁵I-bFGF was released from a Millicell with a radius of 6 mm placed in the center of a cellular monolayer. Under these conditions, ¹²⁵I-bFGF alone elicited a morphologic change only in those cells that were in contact with the filter or <4 mm from the edge of the filter. In contrast, ¹²⁵I-bFGF released in the presence of heparin stimulated the entire monolayer of a 60-mm dish. No change in cell morphology was observed in experiments in which heparin alone (100 μg/ml) was added to the Millicell (data not shown). Previous work demonstrated that heparin does not increase the ability of bFGF to elicit morphological changes when both are added to the culture medium (Moscatelli, 1987). Subsequent autoradiography of the culture dish indicated that the distribution of radioactivity correlated with the pattern of morphological change. ¹²⁵I-bFGF did not diffuse beyond 4 mm from the edge of the Millicell. In contrast, the diffusion of ¹²⁵I-bFGF in the presence of heparin was limited only by the wall of the culture dish. Therefore, these results suggest that heparin increases the radius of stimulation by bFGF released from a central source by 10-fold compared to the radius of stimulation by bFGF alone.

Table I. ¹²⁵I-bFGF Diffusion in the Presence of Glycosaminoglycans and Proteoglycans Isolated from BCE-Conditioned Medium

Addition	Area of diffusion
	cm²
None	1.4 ± 0.1
DEAE eluate (425 μg/ml)	3.1 ± 0.2
Heparin (50 μg/ml)	3.9 ± 0.1

Heparan sulfate glycosaminoglycans and proteoglycans were isolated from conditioned medium as described in Materials and Methods. The effect of this material on the diffusion of ¹²⁵I-bFGF was assayed using diffusion experiments as described in Fig. 2. The autoradiograms were scanned across four different diameters. The values represent the means and standard deviations of the measurements.

5 mm   10 mm   25 mm
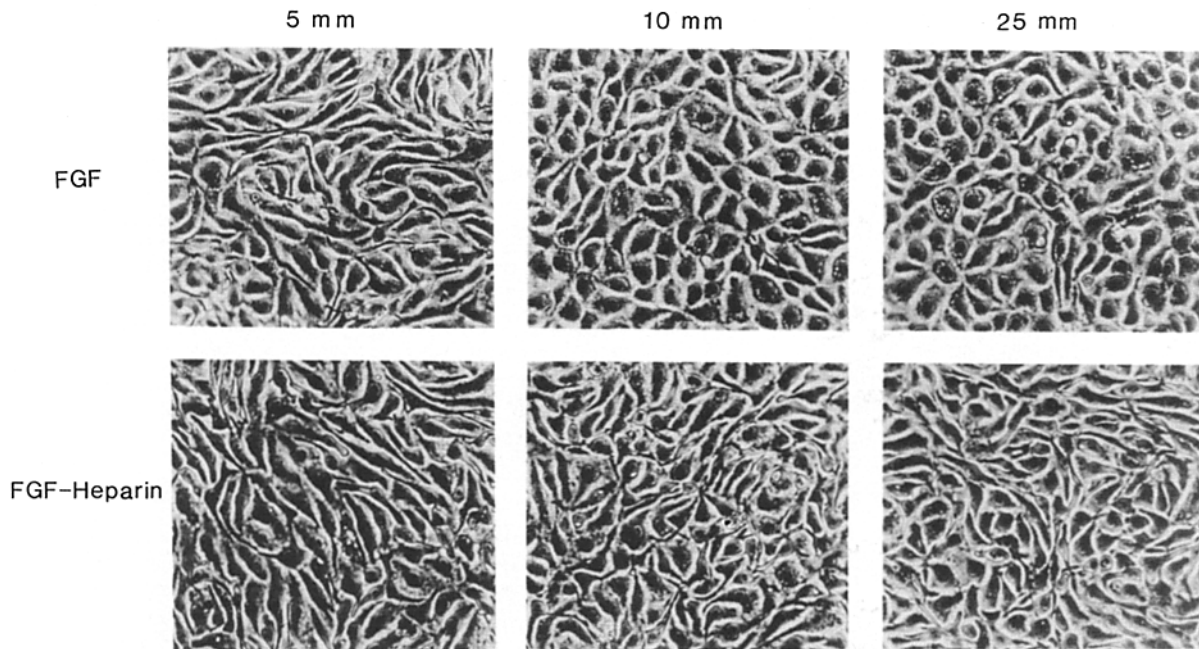
Figure 6. Heparin increases the radius of morphologic alterations induced by bFGF on a cellular monolayer. Diffusion experiments were carried out as described in Fig. 4. After a 36-h incubation, the cells were fixed and stained and the monolayer was partitioned into concentric circles of increasing distance away from the source of bFGF. The monolayer was examined under a light microscope and photographs were taken from each section. The rows of photographs show the effects of 15 ng bFGF diffusing alone and in the presence of 10 μg/ml heparin. The distance from the center of the monolayer is indicated on top of each column of photographs.

Discussion

Many characteristics of the bFGF molecule suggest that it remains cell and/or ECM-associated in vivo. (*a*) bFGF is one of the few known growth factors whose cDNA does not code for a classical signal sequence (Abraham et al., 1986*a,b*). It is thought not to be actively secreted by cells and the only mechanism of bFGF release thus far documented is cell death or injury (Gajdusek and Carbon, 1989; McNeil et al., 1989). (*b*) bFGF has a high positive charge (Lobb et al., 1986*a*) and may therefore interact with negatively charged matrix molecules abundant in the extracellular environment. (*c*) bFGF interacts strongly with heparin (Gospodarowicz et al., 1984; Shing et al., 1984) and has been demonstrated to bind specifically and avidly (K_D = 2 nM) to heparan sulfates in the ECM (Moscatelli, 1987). (*d*) bFGF has been shown to bind to mouse embryonic and Engelberth Holm Swarm sarcoma basement membranes (Jeanny et al., 1987; Vigny et al., 1988) and has been isolated from the basement membrane of cultured endothelial cells (Vlodavsky et al., 1987*a*) and bovine cornea basement membrane (Folkman et al., 1988). Conversely, bFGF has not been purified successfully from serum or culture medium (Gauthier et al., 1987; Vlodavsky et al., 1987*b*), indicating the absence of significant amounts of soluble bFGF. Thus, the vast majority of bFGF in an organism is either intracellular or bound to ECM.

The data presented in this paper support the conclusion that bFGF added exogenously to a cell monolayer associates with the ECM. In these experiments, exogenous bFGF was released from a well cut in an agarose gel or a from a Millicell. Although the mechanism by which bFGF is released in vivo remains unknown, bFGF released from cells by injury has been shown to associate predominantly with the ECM (Gajdusek and Carbon, 1989). Our results suggest that bFGF released from a source would be bound by immobilized anionic molecules in the matrix in the immediate vicinity of the location of release. Yet, it is difficult to understand how this growth factor, bound to the matrix adjacent to its point of release, could stimulate cells at distant sites. In particular, how can bFGF bound to the matrix of the cells which synthesize it stimulate angiogenesis in vascular endothelial cells some distance away?

Several groups (Nakajima et al., 1981; Bar-Ner et al., 1985; Mignatti et al., 1986) have explored the importance of matrix solubilization and degradation preceding the onset of tumor growth and metastasis. Baird and Ling (1987) have shown that heparan sulfate degradation decreases the binding of bFGF to the ECM. They proposed that such degradation might mobilize growth factor stored in the matrix. Other work has demonstrated that matrix-bound bFGF retains its biological activity (Flaumenhaft et al., 1989; Presta et al., 1989) and that bFGF-heparan sulfate complexes released from the ECM by plasmin degradation are capable of stimulating vascular endothelial cells (Saksela and Rifkin, 1990). The mobilized bFGF-heparan sulfate complex is not only active, but it is also protected from protease degradation by plasmin (Saksela et al., 1988; Sommer and Rifkin, 1988). Thus, the ECM potentially acts as a reservoir of bound bFGF

that can be released by enzymatic degradation as a complex that is active and protected from proteolytic degradation.

The results presented here suggest an additional property of the bFGF-heparan sulfate complex released from the ECM: the enhanced ability to diffuse in the extracellular environment. We have demonstrated that heparin increases the radius of diffusion of ^{125}I-bFGF in agarose and fibrin gels, and on a cellular monolayer. Heparan sulfates appear to have a similar, though less pronounced, effect to heparin on the diffusion of bFGF. Saksela et al. (1988) have shown that ~2–5% of heparan sulfates bind bFGF as strongly as heparin. We suggest that heparan sulfate proteoglycan fragments released by proteolytic degradation of the proteoglycan core protein may act as binding molecules that enable bFGF to diffuse from a point source to a vascular supply to stimulate angiogenesis. The masking of the heparin-binding site of bFGF in such complexes allows the growth factor to remain in the soluble phase rather than binding to the insoluble ECM. Thus, even though the complex has a larger size than free bFGF, it diffuses further because of greater partitioning into the fluid phase. It may be a bFGF-heparan sulfate complex, rather than bFGF alone, which stimulates angiogenesis in vivo.

An interesting set of observations that are in accord with the model relate to the progression of malignant melanomas. Malignant melanomas <0.7 cm in thickness can remain quiescent for years (Breslow, 1970). Such lesions, however, can grow rapidly, become invasive, and metastasize once they become vascularized (Srivastava et al., 1986). Though the stimulus for angiogenesis is not known, bFGF has been found in melanoma cell lysates (Lobb et al., 1986b; Moscatelli et al., 1986) and autocrine production of bFGF has been implicated in the pathophysiology of malignant melanoma (Halaban et al., 1987). Furthermore, both mast cell activity (Starkey et al., 1988) and increased proteolytic activity at the tumor site (Nakajima, 1981; Hearing et al., 1988) have been documented in the transition of melanoma to a highly malignant state. It is possible that heparin, secreted by mast cells (Kessler et al., 1976; Azizkhan et al., 1980), or soluble heparan sulfates, released from matrix by hydrolysis (Nakajima et al., 1981; Baird et al., 1987; Saksela et al., 1988), mobilize bFGF bound to the matrix at the tumor site enabling bFGF to diffuse to a blood supply and stimulate angiogenesis. Recent studies using mast cell-deficient mice injected with melanoma cells demonstrate that the angiogenic response is slower and less intense and the number of metastases lower in these mice than in wild-type mice (Starkey et al., 1988). The vascularization of melanomas in mast cell-deficient mice may occur later because bFGF bound to matrix is not mobilized as readily in these mice as in mice with normal, heparin-secreting mast cells.

Thus, while bFGF has properties that make it an effective angiogenic stimulator in vitro, it seems likely that bFGF is unable to diffuse freely in its microenvironment in vivo. The ability of an angiogenic factor to diffuse to its target vascular supply, however, is essential for the factor to be active. It is possible that bFGF acquires this diffusion property by associating with heparin secreted by mast cells or by binding heparan sulfates in the ECM and being solubilized as a bFGF-heparan sulfate complex.

References

Abraham, J. A., A. Mergia, J. L. Whang, A. Tumolo, J. Friedman, K. A. Hjerrild, D. Gospodarowicz, and J. C. Fiddes. 1986a. Nucleotide sequence of a bovine clone encoding the angiogenic protein, basic fibroblast growth factor. Science (Wash. DC). 233:545–548.

Abraham, J. A., J. L. Whang, A. Tumolo, A. Mergia, J. Friedman, D. Gospodarowicz, and J. C. Fiddes. 1986b. Human basic fibroblast growth factor: nucleotide sequence and genomic organization. EMBO (Eur. Mol. Biol. Organ.) J. 5:2523–2528.

Azizkhan, R. G., J. C. Azizkhan, B. R. Zetter, and J. Folkman. 1980. Mast cell heparin stimulates migration of capillary endothelial cells in vitro. J. Exp. Med. 152:931–944.

Baird, A., and N. Ling. 1987. Fibroblast growth factors are present in the extracellular matrix produced by endothelial cells in vitro: implications for a role of heparinase-like enzymes in the neovascular response. Biochem. Biophys. Res. Commun. 142:428–435.

Bar-Ner, M., M. D. Kramer, V. Schirrmacher, R. Ishai-Michaeli, Z. Fuks, and I. Vlodavsky. 1985. Sequential degradation of heparan sulfate in the subendothelial extracellular matrix by highly metastatic lymphoma cells. Int. J. Cancer. 35:483–491.

Breslow, A. 1970. Thickness, cross-sectional areas, and depth of invasion in the prognosis of cutaneous melanoma. Ann. Surg. 172:902–908.

Bitter, T., and H. M. Muir. 1962. A modified uronic acid carbazole reaction. Anal. Biochem. 4:330–334.

Burgess, W. H., and T. Maciag. 1989. The heparin-binding (fibroblast) growth factor family of proteins. Annu. Rev. Biochem. 58:575–606.

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 and DNA synthesis. J. Cell. Physiol. 140:75–81.

Folkman, J., E. Merler, C. Abernathy, and G. Williams. 1971. Isolation of a tumor factor responsible for angiogenesis. J. Exp. Med. 133:275–288.

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.

Folkman, J., M. Klagsbrun, J. Sasse, M. Wadzinski, D. Ingber, and I. Vlodavsky. 1988. A heparin-binding angiogenic protein—basic fibroblast growth factor—is stored within basement membrane. Am. J. Pathol. 130:393–400.

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.

Gospodarowicz, D., and J. Cheng. 1986. Heparin protects basic and acidic FGF from inactivation. J. Cell. Physiol. 128:475–484.

Gospodarowicz, D., R. Gonzalez, and D. K. Fujii. 1983. Are factors originating from serum, plasma, or cultured cells involved in the growth-promoting effect of the extracellular matrix produced by cultured bovine corneal endothelial cells? J. Cell. Physiol. 114:191–202.

Gospodarowicz, D., J. Cheng, G. M. Lui, A. Baird, and P. Bohlen. 1984. Isolation of brain fibroblast growth factor by heparin-Sepharose affinity chromatography: identity with pituitary fibroblast growth factor. Proc. Natl. Acad. Sci. USA. 81:6963–6967.

Gospodarowicz, D., N. Ferrara, L. Schweigerer, and G. Neufeld. 1986a. Structural characterization and biological function of fibroblast growth factor. Endocr. Rev. 8:95–114.

Gospodarowicz, D., G. Neufeld, and L. Schweigerer. 1986b. Fibroblast growth factor. Mol. Cell. Endocrinol. 46:187–204. 775–781.

Halaban, R., S. Ghoshi, and A. Baird. 1987. bFGF is the putative natural growth factor for human melanocytes. In Vitro Cell. Dev. Biol. 23:47–52.

Hearing, V. J., L. W. Law, A. Corti, E. Appella, and F. Blasi. 1988. Modulation of metastatic potential by cell surface urokinase of murine melanoma cells. Cancer Res. 48:1270–1278.

Jeanny, J. C., N. Fayein, M. Moenner, B. Chevallier, D. Barritault, and Y. Courtois. 1987. Specific fixation of bovine brain and retinal acidic and basic fibroblast growth factors to mouse embryonic eye basement membranes. Exp. Cell Res. 171:63–75.

Joseph-Silverstein, J., and D. B. Rifkin. 1987. Endothelial cell growth factors and the vessel wall. Semin. Thromb. Hemostasis. 13:504–513.

Kessler, D. A., R. S. Langer, N. A. Pless, and J. Folkman. Mast cells and tu-

mor angiogenesis. *Int. J. Cancer.* 18:703–709.

Klagsbrun, M., and Y. Shing. 1985. Heparin affinity of anionic and cationic capillary endothelial cell growth factors: analysis of hypothalamus-derived growth factors and fibroblast growth factors. *Proc. Natl. Acad. Sci. USA.* 82:805–809.

Lobb, R., J. Sasse, R. Sullivan, Y. Shing, P. D'Amores, J. Jacobs, and M. Klagsburn. 1986a. Purification and characterization of heparin-binding endothelial cell growth factors. *J. Biol. Chem.* 261:1924–1928.

Lobb, R. R., J. W. Harper, and J. W. Fett. 1986b. Purification of heparin-binding growth factors. *Anal. Biochem.* 154:1–14.

McNeil, P. L., L. Muthukrishnan, E. Warder, and P. D'Amore. 1989. Growth factors are released by mechanically wounded endothelial cells. *J. Cell Biol.* 109:811–822.

Mignatti, P., E. Robbins, and D. B. Rifkin. 1986. Tumor invasion through the human amniotic membrane: requirement for a proteinase cascade. *Cell.* 47:487–498.

Montesano, R., P. Mouron, and L. Orci. 1985. Vascular outgrowths from tissue explants in fibrin or collagen gels: a simple in vitro model of angiogenesis. *Cell. Biol. Int. Rep.* 9:869–875.

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., 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.

Nakajima, M., T. Irimura, D. T. DiFerrante, N. DiFerrente, and G. Nicolson. 1981. Rates of heparin sulfate degradation correlate with invasive and metastatic activities of B16 melanoma sublines. *J. Cell Biol.* 91:119a.

Neufeld, G., and D. Gospodarowicz. 1985. The identification and partial characterization of the fibroblast growth factor receptor of baby hamster kidney cells. *J. Biol. Chem.* 260:13860–13868.

Nicosia, R. F., R. Tchao, and J. Leighton. 1983. Angiogenesis-dependent tumor spread in reinforced fibrin clot culture. *Cancer Res.* 43:2159–2166.

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.

Presta, M., J. A. M. Maier, M. Rusnati, and G. Ragnotti. 1989. Basic fibroblast growth factor is released from endothelial extracellular matrix in a biologi-

cally active form. *J. Cell. Physiol.* 140:68–74.

Rifkin, D. B., and D. Moscatelli. 1989. Recent developments in the cell biology of basic fibroblast growth factor. *J. Cell Biol.* 109:1–6. 6:4060–4066.

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.* In press.

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.

Shing, Y., J. Folkman, C. Butterfield, J. Murray, and M. Klagsbrun. 1984. Heparin affinity: purification of a tumor-derived capillary endothelial cell growth factor. *Science (Wash. DC).* 223:1296–1299.

Smith, J. C., J. P. Singh, J. S. Lillquist, D. S. Goon, and C. D. Stiles. 1982. Growth factors adherent to cell substrate are mitogenically active in situ. *Nature (Lond.).* 296:154–156.

Sommer, A., and D. B. Rifkin. 1989. Interaction of heparin with human basic fibroblast growth factor: protection of the angiogenic protein from proteolytic degradation by a glycosaminoglycan. *J. Cell Physiol.* 138:215–220.

Srivastava, A., P. Laider, L. E. Hughes, J. Woodcock, and E. Shedden. 1986. Neovascularization in human cutaneous melanoma: a quantitative morphological and doppler ultrasound study. *Eur. J. Clin. Oncol.* 22:1205–1209.

Starkey, J. R., P. K. Crowle, and S. Taubenberger. 1988. Mast-cell-deficient W/Wv mice exhibit a decreased rate of tumor angiogenesis. *Int. J. Cancer.* 42:48–52.

Uhlrich, S., O. Lagenete, M. Lenfant, and Y. Courtois. 1986. Effect of heparin on the stimulation of non-vascular cells by human acidic and basic FGF. *Biochem. Biophys. Res. Commun.* 137:1205–1213.

Vigny, M., M. P. Ollier-Hartmann, M. Lavigne, N. Fayein, J. C. Jeanny, M. Laurent, and Y. Courtois. 1988. Specific binding of basic fibroblast growth factor to basement membrane-like structures and to purified heparan sulfate proteoglycan of the EHS tumor. *J. Cell Physiol.* 137:321–328.

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

Vlodavsky, I., R. Fridman, R. Sullivan, J. Sasse, and M. Klagsbrun. 1987b. Aortic endothelial cells synthesize and deposit basic fibroblast growth factor which remains cell associated and platelet-derived growth factor-like protein which is secreted. *J. Cell. Physiol.* 131:402–408.

Yanagishita, M., R. J. Midura, and V. C. Hascall. 1987. Proteoglycans: isolation and purification from tissue culture. *Methods Enzymol.* 138:279–289.