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Modes of FGF release in vivo and in vitro

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Summary

The fibroblast growth factors (FGFs) are a family of polypeptide growth regulators. The prototypes of this family are acidic and basic FGF. Unusual among their characteristics are a high affinity for the glycosaminoglycan heparin and the lack of a signal sequence for secretion. Other members of the FGF family include a number of oncogene products that also display heparin affinity but do possess signal sequences. Results from early tissue culture studies were consistent with the prediction that acidic and basic FGF would not be secreted. Investigators found that virtually no FGF was secreted into conditioned media, instead it remained cell-associated and was deposited into the basement membrane. More recently, however, a number of studies have indicated that a small amount of FGF is 'released' from cells where it is postulated to act as an autocrine regulator. Acidic and basic FGF have been localized in basement membranes both *in vivo* and *in vitro*. The mode of release to this site is also unclear but may be secondary to the mechanisms cited above with soluble FGF becoming bound to heparan sulfate molecules in the extracellular matrix. A number of observations have indicated that matrix-bound FGF is biologically active *in vitro*. There are no data to indicate whether the same is true for FGF bound to basement membranes *in vivo*. In addition to its apparent sequestration in the basement membrane, FGF has also been localized to the surface of a variety of normal and tumor cell types. In particular, endothelial cells have been shown to possess two classes of FGF-binding sites: low abundance, high-affinity receptors that mediate the biological activity as well as high abundance, low affinity binding sites. The physiologic relevance of FGF binding to these low affinity sites is not clear. The possibility of locally high concentrations of heparin released by mast cells, as well as the presence of heparan sulfate-degrading enzymes, suggests that this glycosaminoglycan bound FGF might be released from these binding sites under some circumstances. Cell surface binding of FGF has also been demonstrated *in vivo*; in rabbits plasma levels of the growth factor were shown to be dramatically elevated following intravenous heparinization. Since the FGFs were first noted to lack a signal sequence, cell injury has been suspected to be the most likely route for FGF release *in vivo*. A number of studies using different models of cell injury, including endotoxins and irradiation, have revealed that damaged cells do release FGF. Whether cell death is actually necessary for FGF release was addressed in an experiment in which transient cell injury was caused by cell scraping. These studies revealed that FGF could be released by non-lethal cell injury. The mechanism by which FGF is released to the basement membrane, cell surface and extracellular space is not clear and may be accounted for by cell leakage, cell death, sublethal cell injury, a novel secretion pathway or any combination of these. Identification of the means of FGF release may provide insight into the physiologic role of acidic and basic FGF. Further, it may help to elucidate the mode of release of other biologically active molecules known not to contain signal sequences, including interleukin 1 and platelet-derived endothelial cell growth factor.

The fibroblast growth factors (FGFs) are a family of growth regulatory polypeptides. They have been shown to have a variety of effects on a number of cells; acting as mitogens in some cases, e.g. vascular endothelial cells [1], and stimulating differentiation of others, e.g. neural cells [2, 3]. The prototypes in this family are acidic FGF (aFGF) and basic FGF (bFGF). One of the most intriguing characteristics of the FGFs is their ability to bind strongly to the glycosaminoglycan heparin [1]. This heparin-binding has provided a very powerful purification method for these growth factors [1, 4, 5]. The FGFs are widely distributed, they have been found in virtually every tissue that has been investigated to date. The range of distribution of bFGF appears to be broader than that of aFGF, but this difference may simply be due to the fact that bFGF has been more intensively studied. A great deal of progress has been made in the study of these growth factors over the past five years and the general topic of fibroblast growth factors has been well-covered in a number of recent reviews [6–9].

One of the most perplexing pieces of information with respect to the FGFs is that both acidic and basic FGF lack a consensus signal peptide for secretion [10, 11]. The lack of a signal sequence suggests that the proteins would not be secreted by conventional means. How can FGF act if it has no way out of the cells that synthesize it? The goal of this review is to assemble many of the observations that relate to the release and/or accessibility of FGF and, using this information, to speculate on possible mechanisms for FGF release.

Other members of the FGF family include a number of oncogene products. Unlike acidic and basic FGF, these proteins contain a signal sequence that mediate their secretion [12]. It is possible that the transforming ability of these factors is due to the fact that they are secreted whereas their non-oncogenic counterparts, acidic and basic FGF, are not secreted.

FGF is not secreted?

The expectation that FGF would not be released from cells by conventional means was borne out by early studies of large vessel, small vessel and corneal endothelial cells which have been shown to synthesize bFGF [13, 14, 15). When the localization of FGF was examined in corneal endothelial cells, a *majority* of the factor was found to remain cell-associated or in the extracellular matrix [14]. Similar studies on capillary endothelial cells demonstrated that nearly all of the bFGF to be cell-associated (these authors did not distinguish between cell- and extracellular matrix-associated bFGF) [15].

In spite of the prediction that no FGF would be found in the conditioned media, Schweigerer and his co-workers did report a measurable amount of bFGF in the conditioned media of adrenal cortex-derived capillary endothelial cells [15]. Studies using neutralizing anti-bFGF antibodies indicated that this low level of growth factor does, in fact, influence endothelial cell growth as the addition of neutralizing antibodies resulted in a small, but consistent, suppression of basal proliferation. These authors postulated that cell lysis or leakage might be responsible for this extracellular bFGF. We have similarly found that addition of neutralizing antibodies against bFGF to growing cultures of capillary endothelial cells results in a moderate (20–35%) but reproducible inhibition of their baseline growth (Fig. 1) [16].

The known lack of a signal sequence and these two early studies in which little or no bFGF was detected in the conditioned media of endothelial cells, has led most researchers to believe that no FGF 'escapes' or 'is released' from cells under normal circumstances. However, there is a growing body of information that suggests that this is not entirely true. Sato and his co-workers used Western blot analysis to study the presence of bFGF in serum-free conditioned media from both bovine corneal endothelial cells and a human astrocytoma cell line [17]. The release of FGF by the astrocytoma cell line was between 15 and 50-fold higher than the endothelial cells under various culture conditions. For both cell lines the FGF release was found to be density-dependent. In sparse cells there was a detectable amount of FGF in the media (levels were not quantified in this study). However, as the cells reached confluence, the level of immunoreac-

tive bFGF in the conditioned media was dramatically reduced. These authors suggest that the decrease of FGF in the conditioned media reflects adsorption of the factor to the extracellular matrix but do not speculate on a mechanism for the release. Another study which suggests that bFGF may be 'continuously released by bovine aortic endothelial cell cultures', reported that the migration of endothelial cells following injury *in vitro* could be inhibited by the addition of neutralizing antibodies against bFGF as well as by the addition of protamine sulfate or suramin, compounds that are known to block FGF binding to its receptor [18].

Central to the interpretation of these two studies was the concept that FGF may be acting in an autocrine manner. However, without exception, these endothelial cell functions (proliferation and migration) can be further stimulated by the addition of exogenous FGF, suggesting that if there is an autocrine role for FGF it is in maintaining baseline functions. Interestingly, Sato and Rifkin [18] also report that neutralizing antibodies against bFGF can block the migration of BHK-21 and NIH 3T3 cells following injury. Since others have demonstrated that these cells [19, 20] do not normally synthesize bFGF the interpretation of these results is unclear. It may be that the two cell types do make some low level of bFGF which is undetectable by the available means.

Additional evidence for an autocrine role for bFGF comes from transfection studies by Neufeld and co-workers. When BHK21 cells, which do not normally express bFGF, were transfected with the cDNA for bFGF, they proliferated rapidly even in serum-free media [19]. These authors were not confident that these results reflected bFGF secretion. On one hand, the conditioned media produced by these cells did contain mitogenic activity which bound to heparin Sepharose and was neutralized by the addition of antibodies against bFGF. On the other hand, the biological activity in the conditioned media accounted for less than 1% of the total activity and the addition of neutralizing antibodies did not block the proliferation of the transfected cells. The authors point out that the lack of an effect by neutralizing antibodies does not necessarily indicate absence of secretion. An alternate explanation might be that FGF, once released, binds extremely rapidly to its receptors and becomes inaccessible to the neutralizing antibody. The fact that protamine was capable of blocking proliferation of the transfected cells supports this possibility. In general, however, the data provided by this study do not provide conclusive evidence one way or the other with respect to the release of FGF and its site of action.

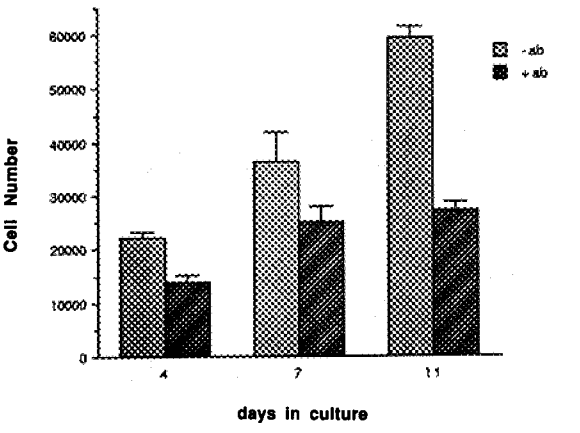

Fig. 1. Effect of neutralizing anti-bFGF antibodies on baseline capillary endothelial cell proliferation. Adrenal cortical capillary endothelial cells were plated at sparse densities (5,000 cell/cm^2) into gelatin-coated wells in Dulbecco' modified Eagle's medium with 10% calf serum and allowed to attached overnight. The following day the media were changed and anti-bFGF IgG (prepared in collaboration with Dr Ira M. Herman, Tufts Medical School, Boston, MA) (1:125) were added. The cells were counted electronically on the fourth and seventh days and the remaining cultured were refed with additional antibodies. The presence of the antibodies suppresses a significant proportion of baseline growth.

Some insight into the interpretation of this study may be provided by a separate study by Rogelj and co-workers in which a cDNA encoding for bovine bFGF linked to the amino-terminus immunoglobulin signal peptide was transfected into NIH 3T3 cells [20]. The parental 3T3 cell line makes very low levels of bFGF but does express cell surface receptors. Following transfection, the growth pattern of the cells was markedly changed. The transfectants grew as large aggregates whereas the controls grew as a contact-inhibited monolayer. Furthermore, the transfected cells, unlike the parental cells, were

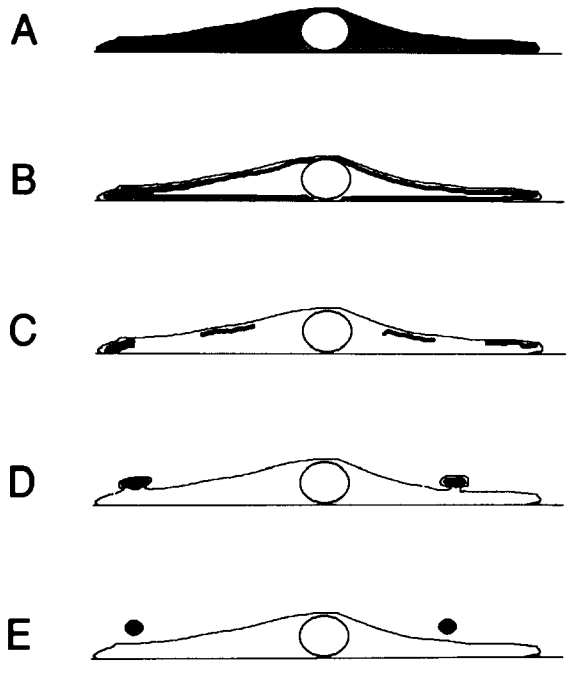

Fig. 2. A schematic diagram of a novel pathway for the release of proteins lacking signal sequences. (a) The protein is initially distributed throughout the cytoplasm, (b) becomes concentrated next to the plasma membrane where (c) it accumulates in specific regions. (d) These areas of the plasma membrane are then evaginated and (e) form extracellular vesicles that are enriched for the protein. Subsequent disruption of the vesicles release the protein to the media where it can bind to the cell surface or basement membrane. Taken from Cooper and Baronades (J Cell Biol 110: 1681–1693, 1990) with permission from Rockefeller Press.

tumorigenic. Interestingly, even these cells, whose FGF contained a signal peptide, did not appear to secrete FGF into the conditioned media; rather, the growth factor remained cell-associated. In light of the observations by Neufeld and co-workers, it is possible that these cells do secrete bFGF, but that it is rapidly bound to cell surface binding sites which include both high affinity receptors as well as low affinity heparan sulfate molecules. There are no data in this paper to support or deny this interpretation. On the other hand, data from Moscatelli and Quattro suggest that surface-bound bFGF is important for the transformed phenotype [21]. In these studies transfection of NIH 3T3 cells with a bFGF cDNA led to a morphological transformation and down-regulation of bFGF receptors. These alterations were reversed by the addition of suramin, suggesting that interaction of bFGF with its surface receptor is necessary to observe the transformed phenotype.

Possible pathways for the release of the FGFs include cell leakage and cell death (see 'Release of bFGF by Cell Injury' below). However, an alternate mode of FGF release is suggested by a very recent study which reports the export of a lectin from C2 mouse muscle cells by plasma membrane evaginations [22]. This lectin, like FGF, lacks a signal sequence and has been detected in the extracellular matrix. Immunohistochemical studies have revealed that the lectin becomes concentrated in patches beneath the plasma membrane, which then evaginate resulting in extracellular vesicles that contain the lectin (Fig. 2). The authors speculate that the material released from disrupted extracellular vesicles is then free to bind to the cell surface and/or the extracellular matrix. Release of the lectin in this system appears to be polarized to the apical surface. However, it would seem that such a mechanism might also release materials directly to the extracellular matrix. This novel mechanism for protein release is appealing as a mechanism for FGF release as it is consistent with the fact that FGF is found to be sequestered both on the cell surface and in the extracellular matrix.

FGF is localized in basement membranes in vitro

Studies that documented FGF deposition into the extracellular matrix by cultured cells provided an explanation for an early observation made by Gospodarowicz and his co-workers [23]. They found that basement membranes synthesized by corneal endothelial cells could support the growth of clonal densities of vascular endothelial cells which would otherwise require the addition of growth factors such as FGF. The corneal endothelial cell-synthesized basement membrane was also shown to induce the differentiation (neurite outgrowth) of PC12 cells [24]. A likely reason for the growth- and differentiation-promoting effects of this matrix was the presence of bFGF. This was most conclusively demonstrated in a recent series of studies by Rogelj

and colleagues [25]. In these experiments endothelial cells and PC12 cells were plated onto extracellular matrix in the presence of polyclonal antibodies against bFGF (Fig. 3). Both endothelial cell proliferation and neurite outgrowth by PC12 cells were inhibited in the presence of the antibody. In a second series of elegant studies a cell line known not to make FGF was allowed to produce an extracellular matrix; the matrix did not support the two cell functions. After transfection with a cDNA for bFGF, the cells produced an extracellular matrix that was able to support both endothelial cell growth and PC12 cell differentiation. The effect of this matrix was inhibited by the addition of anti-bFGF antibodies. These studies provide definitive evidence that bFGF is the active component in the extracellular matrix responsible for growth and differentiation-promoting actions of the matrix, and further indicate that bFGF in the extracellular matrix is present in a biologically active form. Not surprisingly, the extracellular matrix associated-FGF appears to be bound to heparan sulfate molecules as it is released by heparin-like compounds and heparitinase [26]. Acidic FGF has also been localized in basement membranes; it has been shown to be deposited in the basal lamina of neonatal cardiac myocytes in tissue culture [27]. These observations of FGF binding to the extracellular matrix led to the suggestion that the matrix might serve as a reservoir for these growth regulators. In this location they might then act through both paracrine and autocrine mechanisms to affect a variety of cell functions, including neural differentiation, myocyte differentiation, and the process of angiogenesis.

	EC [^{3}H]thymidine incorporation		
	Plastic	Plastic + FGF	ECM
	cpm × 10^{-3}	*cpm* × 10^{-3}	*cpm* × 10^{-3}
Control	8.3	56.5	84.0
+ Anti-bFGF	6.8	9.7	20.5
+ Nonimmune IgG	8.1	55.3	82.5

Bovine aortic ECs were seeded (5×10^2 cells/cm^2) in DME plus 10% calf serum into regular and CE-ECM-coated wells of 24-well tissue culture plates. Recombinant bFGF (2 ng/ml), rabbit anti-bFGF IgG (40 μg/ml), or nonimmune rabbit IgG were added to some of the ECM-coated wells on days 1 and 3 after seeding. [^{3}H]Thymidine (2.5 μCi/well) was added on day 5 for 2 h and the amount of thymidine incorporation into TCA-insoluble material was determined. Each data point represents the mean of six determinations and the variation between different determinations did not exceed 15% of the mean.

Fig. 3. Effect of anti-bFGF antibodies on bFGF- and ECM-induced stimulation of EC proliferation. Taken from Rogelj *et al.* (J Cell Biol 109: 823–831, 1989) with permission from Rockefeller Press.

FGF is localized in basement membranes *in vivo*

The *in vitro* observations of the localization of the FGFs in basement membrane were thought by some to be artifacts of tissue culture. However, demonstration of the FGFs in basement membranes *in vivo* has added credibility to the hypothesis that the basement membrane acts as a physiologic reservoir for these growth factors. Basic FGF was first shown to be sequestered in the basement membrane of the bovine cornea [28]. Heparin affinity chromatography and immunofluorescence localization revealed bFGF in Descemet's membrane which underlies corneal endothelial cells and in Bowman's membrane upon which the corneal epithelium sits (Fig. 4). Basic FGF has also been localized to the extracellular matrix of mouse skeletal muscle [29]. Interestingly the mdx mouse, a murine model of muscular dystrophy in which muscle undergoes continuous regeneration, displays elevated levels of FGF in its extracellular matrix. The authors suggest that the absence of dystrophin in the mouse fiber has some causal role in the increase of basement membrane-associated FGF. Furthermore, they speculate that the increased level of FGF in the basal lamina could be responsible for the continuous fiber regeneration that is observed.

The mechanism by which acidic and basic FGF find their way to the basement membrane in these *in vivo* and *in vitro* situations is not known. It has been suggested that the FGF might 'piggy back' on the nascent heparan sulfate to be transported out of the cell. However, the likelihood of this is not high as heparan sulfate proteoglycan modification occurs in the Golgi complex and the proteoglycan is then exocytosed via membrane-bound vesicles. The fact that FGF lacks a signal sequence makes it unlikely that FGF would be inside of a membrane-bound vesicle and have the opportunity to interact with the glycosaminoglycan. Other explanations

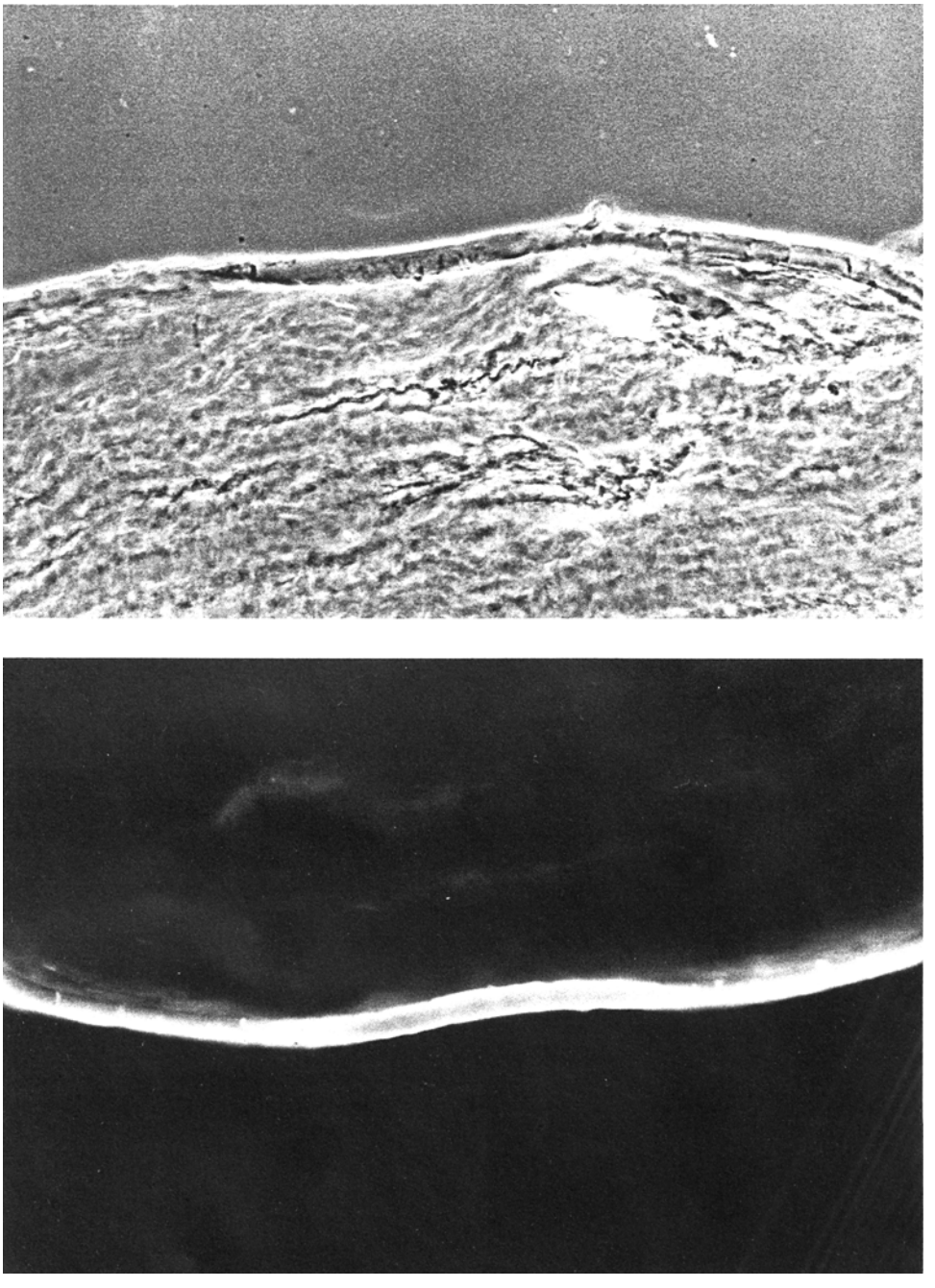

Fig. 4. Immunolocalization of bFGF in Descemet's membrane. Phase (top) and immunofluorescence (bottom) micrographs of frozen sections of normal bovine cornea that were stained with antibodies directed against an internal portion (amino acids 33–43) of bFGF. Micrographs courtesy of Dr. D. Ingber, Children's Hospital, Boston, MA.

suggest a more 'passive' mode for the deposition of FGF into the extracellular matrix. A low level of cell death or leakage would introduce FGF into the extracellular space where it might then be bound by heparan sulfate in the basement membrane. Alternatively, it is known that as cells migrate they leave behind 'bits' of cytoplasm. Since FGF appears to be localized in the cytoplasm, the continuous deposition of FGF-containing cytoplasm might lead to an accumulation of extracellular FGF. Another possible mode for the deposition of FGF into the basement membrane from cytoplasmically localized material involves the evagination of plasma membranes vesicles [22] (see 'Is FGF Secreted?' and Fig. 2).

Whatever the mode of FGF deposition into the basement membrane, it is clear that the matrix-bound FGF is biologically active. Yet, it is not known whether FGF must be released from the basement membrane or whether it can act in its bound state. The fact that basement membrane-bound bFGF is biologically active was demonstrated in the *in vitro* studies referred to above in which corneal endothelial cell-derived basement membrane was shown to support clonal growth of endothelial cells [22] as well as neural differentiation [22]. More recent studies in which bFGF bound to matrix was shown to induce long-term stimulation of both plasminogen activator production and DNA synthesis by endothelial cells [30, 31] have also demonstrated the biological activity of matrix-bound FGF. Further, matrix-associated FGF may be stabilized as it appears that heparin is able to protect the FGFs from inactivation by proteases and heat [32, 33].

In spite of its known biological activity, the presence of FGF in basement membranes *in vivo* does not appear to stimulate the overlying endothelial and epithelial cells to proliferate. These observations suggest that either the FGF is not accessible under these circumstances or that there is an inhibitor(s) present that can override the actions of the FGF. It should be noted that in the *in vitro* studies cited the cells that have synthetized the matrix are removed (usually by Triton or urea treatment) before the matrix is assayed for its effects. It is possible that this manipulation alters FGF accessibility. On the other hand, if release is a prerequisite for its activity there do seem to be means for FGF release; *in vivo* FGF has been shown to leach from matrices under some circumstances [31], and can be released from the basement membrane in a biologically active form by heparitinase and heparin-like molecules [26].

FGF is localized on cell surfaces *in vitro* and *in vivo*

In addition to basement membrane-bound FGF, there appears to be a significant population of FGF molecules bound to heparan sulfate on cell surfaces. It is well known that virtually all mammalian cells display heparan sulfate proteoglycans on their cell surfaces [34]. These sulfated glycosaminoglycans have been shown to bind a variety of molecules including lipoprotein lipase [35, 36], diamine oxidase [37] and superoxide dismutase [38]. Moscatelli [39] has used Scatchard analysis to determine the number of bFGF binding sites per cell and their affinities. He estimates that bovine capillary endothelial cells have approximately one million binding sites per cell. The majority of these sites have a Kd of 2 nM and are referred to as low affinity. Conversely, there are less than 10,000 high affinity binding sites on the same cells. These receptors have an affinity of about 20 pM and have been shown to mediate the biological activity of bFGF. Since binding to the low affinity sites can be competed with heparin and blocked by heparitinase treatments, it is highly likely that these low affinity binding sites represent heparan sulfate molecules on the endothelial cell surface.

FGF release by heparin has been postulated to be relevant in tumor vascularization. Mast cells which are associated with tumors [40] secrete heparin which may, in turn, release FGF from the surface of tumor cells. In support of this hypothesis, we have demonstrated that the addition of increasing levels of heparin to tumor cells (SK hepatoma) in suspension leads to the release of stimulatory activity that has been characterized as bFGF-like (Fig. 5) [41]. The local release of bFGF from tumor cells might then act to stimulate angiogenesis. The presence of mast-cell derived heparin might also

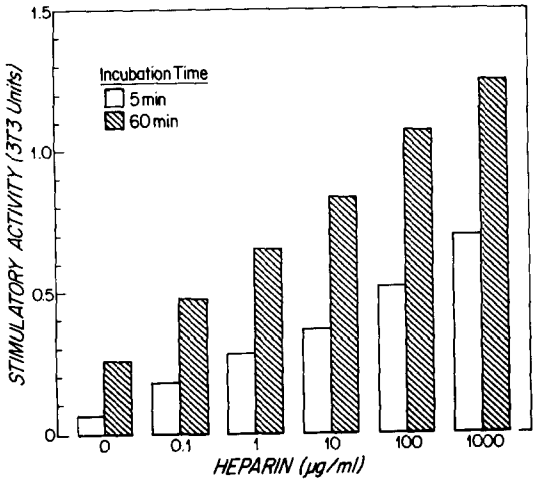

Fig. 5. Release of bFGF-like activity from suspensions of SK hepatoma cells by increasing concentrations of heparin. SK hepatoma cells grown in suspension cultures were divided into aliquots and the indicated concentrations of heparin were added. The cells were then removed by centrifugation 5 min (open bars) or 60 min (hatched bars) after heparin addition and the supernatant assayed for growth stimulatory activity in a 3T3 cell thymidine incorporation assay. The addition of heparin to the hepatoma cells resulted in a dose-dependent release of activity in the media. Subsequent studies indicate that this activity is bFGF-like (Frissora and D'Amore, manuscript in preparation).

prolong the FGF half-life by protecting it against protease actions [32, 33]. A similar mechanism may act in the induction of collateralization as we have shown that heparin is effective in increasing the levels of FGF-like molecules released from myocardial tissue [42].

In spite of the fact that heparin-like molecules are known to be components of cell surfaces and the FGFs display high affinity for heparin, there is little evidence for the association of FGF with cell surfaces *in vivo*. We have recently conducted an *in vivo* study which we feel demonstrates the localization of FGF on the surface of the cells of the vascular wall [43]. In these studies rabbits were infused with anticoagulant heparin and plasma growth-stimulatory activity was measured before and after heparinization. Following the administration of heparin, the level of 3T3 cell stimulatory activity in the media was dramatically increased. In addition, animals that had been administered heparin (to remove endogenous FGF) were then given iodinated bFGF which was rapidly 'cleared from the plasma'. Readministration of heparin released this radiolabeled FGF back into the circulation. These observations suggest that the vascular endothelium, which is known to be lined by heparin-like molecules, has bFGF bound to its surface.

The physiologic relevance of this heparin-mediated release of FGF into the circulation is not clear. In one series of studies, bFGF at a dose of 2.5 ng/kg/min, was infused into rats. An autoradiographic study of ^{3}H-thymidine incorporation into the vascular endothelial cells and smooth muscle cells of these animals revealed no change in the labelling index, indicating that these vascular cells are 'refractory to this factor when it is administered intravascularly' [44]. On the other hand, Linder and co-workers [45] demonstrated that the infusion of bFGF into rodents following de-endothelialization by balloon catheterization resulted in a much more rapid endothelialization of the intimal surface than the untreated controls. In addition, dramatic smooth muscle cell hyperplasia was noted in these FGF-treated animals. Taken together, these two sets of observations suggest that if the vasculature is somehow compromised, either by traumatic or perhaps even more subtle injury, then plasma-borne FGF may influence the proliferation of the cells of the vascular wall. If, on the other hand, there is no insult to the integrity of the vascular wall, then circulating FGF is without an effect.

How endothelial cells remain 'refractile' to this potent mitogen is not clear at this time. One possible explanation for the lack of response to FGF may be found in the work of Ingber and his coworkers [46]. They demonstrated that the ability of endothelial cells to respond to FGF could be modulated by plating endothelial cells on different matrix components in the presence of a defined serum-free media. In particular, they noted that when endothelial cells were plated on a laminin matrix they no longer responded to the addition of exogenous bFGF. The authors speculate alterations in cell shape that occur on different matrices determine whether cells respond to FGF receptor binding by signal transduction.

Alternatively, the presence of inhibitory factors might act to suppress cell proliferation in the pres-

ence of excess FGF. Using a tissue culture model we have previously shown that contact between endothelial cells and smooth muscle cells (or pericytes), that is known to occur throughout the vasculature, inhibits the proliferation of endothelium via generation of activated transforming growth factor type β (TGF-β; [47, 48]. We speculate that the growth state of the vascular endothelium is determined by a balance between growth stimulators such as FGF and growth inhibitors such as TGF-β. It is possible that in the mature vessel wall the level of TGF-β is sufficient to override the FGF administered in the above study.

Release of FGF by cell injury

Since the FGFs were first observed to lack a signal sequence, cell injury has been suggested to be the most likely physiologic mode of FGF release *in vivo*. In one study, Gadjusek and Carbon [49] used endotoxin, which is known to be highly toxic for aortic endothelial cells, to demonstrate cellular release of bFGF. Similarly, irradiation has been shown to release FGF-like activity from endothelial cells. Although irradiation was associated with an increase in cell death, it also led to an increase in *de novo* synthesis of the growth factor, reaching a peak 72 hr following radiation treatment [50]. The authors speculate that radiation-induced release of these growth factors may be involved in the pathogenesis of early vascular damage and late fibrosis of radiation damage.

Although it seems clear that injury should be able to release cytoplasmically localized growth factors, it is not clear whether *lethal* cell injury is necessary for growth factor release. We addressed the possibility that *transient, sublethal* injury to cells might be sufficient to release significant amounts of FGF. These studies were based on earlier work by McNeil and Ito [51] which suggested that membrane disruption might be a common occurrence *in vivo*. Their early studies were conducted in the gastrointestinal system where motile events appear to cause frequent injury to cells of the gut. This prompted them to speculate that *in situ* occurrence of plasma membrane wounding followed by resealing might reflect a previously 'unrecognized route for molecular traffic in and out of the cytoplasm'. With this in mind, we conducted studies in which endothelial cell plasma membranes were transiently injured by scraping [52]. Media conditioned by these injured cells were then assayed for the presence of FGF-like activity. Significant levels of FGF-like growth factor activity were found in the conditioned media of cells that had been injured by scraping, but not by cells that were killed by metabolic poisoning. These studies provide strong evidence that transient cell injury is sufficient to release FGF, and as such may represent an alternate mechanism for the secretion of FGF *in vivo*.

Whether cell injury is a physiologically relevant mechanism for FGF release *in vivo* has yet to be demonstrated. In an early study we documented that the level of stimulatory activity released from myocardial tissues (extracted *in vitro*) was positively correlated with creatine phosphokinase level, a measure of cell injury [53]. Later studies identified this activity as a combination of acidic and basic FGF [54]. It is clear that angiogenesis is often (if not always) observed in tissues where there are ischemic events. These include diabetic retinopathy, the development of collateral growth in association with ischemic heart disease, tumor vascularization, and wound healing. Whether cell death and lysis is necessary in these cases to make available sufficient levels of FGF (if FGF is the angiogenic mediator) has not been determined. Further, whether there is a 'place' for more subtle cell injury and whether injured cells may become 'leaky' to the cytoplasmically localized FGF is another unknown. In addition, it appears that FGF may be delivered locally by blood cells such as platelets and macrophages which have been demonstrated to contain a variety of growth factors including FGF [57, 55, 56].

The subcellular localization of FGF in these two cell types has not been determined. However, for reasons that were discussed in detail above, it is unlikely that the FGF would be localized in membrane-bound granules. Yet, since both cell types release nearly their entire cell constituents when activated to do so, it seems that even cytoplasmical-

ly localized FGF would be delivered during platelet and macrophage release. This would provide another mechanism to deliver high concentrations of FGF (and other growth factors) to appropriate sites in a controlled time and manner.

Conclusions

- Neither acidic nor basic FGF have a signal sequence. They are therefore not released by conventional modes of secretion.
- Low, yet detectable, levels of bFGF are reproducibly found in culture media of cells that synthesize bFGF.
- Basic FGF is found associated with the basement membrane both *in vitro* and *in vivo*. It has been shown to be biologically active *in vitro*.
- FGF is associated with low affinity (heparan sulfate) binding sites on the surface of a variety of cells.
- FGF can be released from cell- and matrix-associated sites by heparin, heparan sulfate and heparitinases.
- A number of forms of injury, including sublethal injury, to cultured cells can release FGF.

Key unanswered questions

- Do the two forms of FGF, acidic and basic, perform different functions e.g. in wound healing, embryogenesis or pathologic neovascularization? Do they have differential expression or differing accessibilities?
- What is the mechanism of FGF release *in vivo*? Is injury necessary? If so, does the injury need to be fatal or is sublethal cellular injury a mechanism of FGF release?
- How is FGF deposited into the basement membrane? Is it an active process mediated by some as yet unknown mechanism? Or, is it passive, binding to the matrix-associated heparin-like molecules after non-specific release from cells?
- Is there a physiologic role for the FGF that is bound to the low affinity heparin-like molecules on cell surfaces and in the basement membrane? Do these binding sites act as reservoirs for FGF?
- What is the significance of the fact that some members of the FGF family (that are oncogene products) possess a signal sequence and are secreted?
- Does FGF, in fact, need to be released to effect its actions or can it act intracellularly?
- Why does intraveneously administered FGF have no influence on the proliferation of cells of the intact vascular wall?
- Does FGF have an autocrine role?
- Is transformation in cells that overexpress bFGF due to the action of bFGF intracellularly or at the cell surface?
- What is the subcellular localization of FGF?
- Is a novel secretion pathway, evagination of plasma membranes, a mechanism for FGF release?
- Are platelets and macrophages major routes for FGF delivery *in vivo*?

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