Journal of Cellular Biochemistry 47:201–207 (1991)

PROSPECTS

Release of Basic Fibroblast Growth Factor, an Angiogenic Factor Devoid of Secretory Signal Sequence: A Trivial Phenomenon or a Novel Secretion Mechanism?

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Abstract Basic fibroblast growth factor (bFGF), a potent angiogenesis inducer, lacks a signal sequence. Therefore, it has been proposed that bFGF is primarily released from dead or damaged cells. Other proteins devoid of secretion signals, interleukin 1β (IL-1β) and the muscle lectin L-14, have been shown to be released via exocytosis, a novel secretion pathway independent of the "classic" endoplasmic reticulum-Golgi route. In the light of these findings and of our own recent results, we discuss evidence that bFGF can be released from single, uninjured cells and mediate functions in an autocrine manner. As is the case for IL-1β and L-14, externalization of bFGF may occur via exocytosis, a pathway utilized during development and differentiation.

Key words: bFGF, secretion, exocytosis, IL-1, cell migration, autocrine growth factors

Basic fibroblast growth factor (bFGF) is the prototype member of a family of structurally related polypeptides with growth-regulatory properties [1]. As is the case for several cytokines, the name originally given to this growth factor [2] now appears to be a misnomer, or at least a very incomplete definition. Basic FGF has been found in a number of cultured cell types, normal tissues, and tumors [3–5] and has been shown to modulate functions of cells of mesodermal, endodermal and ectodermal origin [1,5–7]. It acts as a mitogen for some cells, including fibroblasts [2] and endothelial cells [6,7], and induces differentiation in others, such as neural cells [8,9]. As is true for most growth factors, the physiological role(s) of bFGF is/are still unknown. A most intriguing role proposed is that of an inducer of embryonic development [10–15]. In vascular endothelial cells bFGF stimulates a number of functions involved in the formation of blood vessels (angiogenesis). These include cell proliferation, protease production, migration, and invasiveness [6,7,16–18]. Basic FGF is one of the more potent angiogenesis inducers in vivo and in vitro [19,20].

An important property of bFGF is its ability to bind strongly to heparin and heparan sulfate glycosaminoglycans [21–25]. While the high affinity of this interaction has provided a powerful tool for the purification of this growth factor [6,7,26], it may also indicate a physiologically significant role for the binding of bFGF to the glycosaminoglycans of the extracellular matrix. Basic FGF is associated with heparan sulfate proteoglycans in vitro [21,22,24], and is present in basement membranes in vivo [27,28]. The growth factor associated with the extracellular matrix fully retains its activity [29,30]. Moreover, binding to heparin and heparan sulfate glycosaminoglycans protects bFGF from proteolytic degradation [31]. The interaction of bFGF with the extracellular matrix represents an important mechanism for modulating the extracellular activity of this growth factor. Heparan sulfate-deficient CHO cells transfected with the mouse bFGF receptor gene do not bind bFGF. Addition of heparin and heparan sulfate reconstitutes a low-affinity receptor, which is re-

Received July 11, 1991; accepted July 22, 1991.

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quired for bFGF binding to the high affinity membrane receptor [32]. Basic FGF has a pI greater than 9.6; therefore, under physiological conditions it is highly positively charged. When released into the extracellular environment, bFGF will bind to the negatively charged (sulfated) glycosaminoglycans present in the extracellular matrix and remain localized in an insoluble form. Basic FGF-glycosaminoglycan complexes can be released from the matrix by heparitinase and plasmin digestion [33,34]. The interaction of bFGF with glycosaminoglycans neutralizes the positive charge of the growth factor. Thus, in its complex form bFGF will tend to partition into the aqueous phase rather than in the insoluble matrix. Flaumenhaft et al. [35] have shown that bFGF-heparin complexes diffuse more freely than free bFGF in cultures of endothelial cells and exert their activity on cells at a greater distance from the source of the growth factor.

The hypothesis of an extracellular mode of action for bFGF is also supported by the presence of multiple forms of plasma membrane receptors on a variety of cell types [22,36–40]. Other members of the FGF family, namely acidic FGF (aFGF) and hst/k-fgf, appear to share receptors with bFGF, although with different affinities [39,41,42]. Interestingly, in several cell types the number of bFGF receptors per cell seems to be inversely proportional to the intracellular content of the growth factor [22,42]. This apparent down-regulation of the cell receptors has been interpreted as a result of an autocrine mechanism of action. This hypothesis is also supported by the finding that neutralizing antibodies to bFGF alter several properties of bFGF-producing cells, including morphology, growth in soft agar, plasminogen activator synthesis, and cell migration [43,44]. Protamine sulfate and suramin, which block bFGF-receptor interactions, have the same effect [42,45].

These important features of bFGF, which indicate an extracellular role, contrast with a most intriguing molecular characteristic of this growth factor. All forms of the primary translation products of bFGF lack a hydrophobic signal sequence that would direct their release via the "classic" secretory pathway [46,47]. This quite unexpected feature for a growth factor is also shared by other two members of the FGF family, aFGF and int-2 [1], as well as by interleukin 1 (IL-1) [48,49], the platelet-derived endothelial cell growth factor (PD-ECGF) [50], the ciliary neurotrophic factor (CNTF) [51], thymosin, and parathymosin. In contrast, the other members of the FGF family, hst/k-fgf, FGF-5, FGF-6, and KGF, are initially translated with hydrophobic signal sequences and are secreted into the culture medium of transfected cells [1,52–54]. Consistent with the lack of a secretory signal peptide, cell fractionation analysis has revealed very little bFGF associated with vesicular membrane structures. The 18 KDa form of bFGF is localized primarily in the cytosol, while the 22 KDa, 22.5 KDa, and 24 KDa forms are found in the nucleus [55]. Very little or no bFGF is found in the medium of most cells [4,56–59]. In contrast, bFGF immunoreactivity has been observed within the chromaffin granules of bovine adrenal medulla [60].

Since bFGF lacks a secretory signal sequence, cell death or damage have been proposed to be the most likely mechanism for bFGF externalization [58]. Endotoxin and irradiation have been shown to induce release of bFGF or bFGF-like activities from cultured endothelial cells [61–62]. Although it seems clear that cell injury should cause release of cytoplasmically localized growth factors, lethal cell damage does not appear to be necessary for bFGF externalization. By mechanically inducing plasma membrane disruptions, McNeil et al. [63] have shown that transient, "sublethal cell injury" can be a significant route of bFGF release from cultured endothelial cells. In this study the authors also reported that "leakage" of bGFG from migrating endothelial cells may spontaneously occur in a small percentage of the cells in culture. This is interesting in the light of a previous report which showed that motile events can cause frequent injury to cells of the gastrointestinal tract [64]. It has been speculated that the in situ occurrence of plasma membrane wounding may represent a mechanism for molecular traffic in and out of the cytoplasm. In other studies, Galloway et al. [65] have reported that the level of bFGF and aFGF released from myocardial tissues positively correlated with creatinine phosphate level, a measure of cell damage. It should be considered that angiogenesis most often follows ischemic events, which are clearly a cause of cell injury. Such events occur in acute and chronic myocardial ischemia, as well as in diabetic retinopathy, wound healing, and tumor angiogenesis. However, whether cell injury is a

physiologically relevant mode of bFGF release in vivo remains to be determined.

To address this problem we have devised an experimental system to study the effect of neutralizing anti-bFGF IgG on the migration of single, isolated cells. Under these conditions contamination by bFGF derived from cells other than the one being observed can be excluded. For this purpose we have modified the phagokinetic track assay originally described by Albrecht-Buehler [66]. In this assay cells are allowed to migrate on a microscope coverslip coated with colloidal gold. Under dark field illumination the gold particles appear as a homogeneous layer of highly refringent particles on a dark background. A cell migrating on this substrate phagocytizes or pushes aside the gold particles, thus producing a dark track free of refringent particles [66]. Two clones of NIH 3T3 cells transfected with the human bFGF gene, as well as control NIH 3T3 cells transfected with the viral vector alone (which express very low amounts of bFGF) [67], were seeded onto colloidal gold-coated coverslips at a density of 1 cell/coverslip in microculture plates containing 1 coverslip/well. The area of the phagokinetic tracks formed after 16 h incubation at 37°C was measured by an image analyzer. Under these conditions cell motility directly correlated with the amount of bFGF produced by the cells. Washing the cells with suramin to remove potential contaminating bFGF [45] derived from cells lysed or damaged before or during trypsinization did not affect cell migration. On the contrary, addition of recombinant bFGF stimulated cell motility both of the bFGF transfectants and of the control NIH 3T3 cells in a dose-dependent manner. Thus, cell motility could be modulated by the extracellular bFGF concentration. Addition of affinity-purified anti-recombinant bFGF antibody dramatically reduced the motility of the bFGF transfectants to the level observed in control cells transfected with the viral vector alone [Mignatti et al., manuscript submitted for publication]. These results demonstrate that the bFGF produced by a migrating cell is exported into the extracellular milieu and stimulates migration of the same cell, in the absence of any obvious cell damage. While we cannot rule out that release of bFGF may occur only in actively migrating cells or in cells expressing relatively large amounts of bFGF and is not a phenomenon shared by all bFGF-producing cells, these data provide evidence that bFGF is released from viable, apparently uninjured cells and does not derive only from dead or damaged cells. Thus, the mode of action of bFGF appears to be via a "true" autocrine mechanism.

Our finding also raises an interesting point as to the fate of bFGF after it is released from the cell. In our single-cell phagokinesis assay, one cell was allowed to migrate in 1 ml of culture medium for 16 h. The ratio of the cell volume to the volume of medium was extremely small; therefore, it is unlikely that the concentration of bFGF in the culture medium could be in the range of its K_D for the cell receptor(s). Thus, once released, bFGF must be concentrated on or very close to the cell surface. This can be achieved either through binding directly to the plasma membrane receptor(s) and/or through interaction with cell-bound glycosaminoglycans or other cell-surface molecules. Whatever the interaction with the cell surface, bFGF remains accessible to the antibody.

While these results provide evidence that bFGF is released from cells in the absence of apparent cell injury and acts in an autocrine manner, the intimate molecular mechanism(s) by which this occurs remain(s) an unanswered question. A recent report [68] has shown that IL-1β, which also lacks a secretory signal sequence, appears to be present in intracellular vesicles of activated human macrophages, where it is protected from protease digestion. Drugs which block the intracellular transport of secretory proteins, such as interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF α), do not inhibit release of IL-1β. However, IL-1β externalization is blocked by methylamine, low-temperature (18°C), or serum-free conditions, which inhibit endo- and exocytosis [69,70], and is increased by heat-shock or by the calcium ionophore A23187, which stimulate exocytosis [71,72]. Thus, IL-1β appears to be released from activated macrophages via exocytosis, a novel secretion pathway independent of the "classic" ER-Golgi route. This finding is in agreement with previous reports that in cultured macrophages cytosolic blebs or "podosomes" protrude and detach from the cell surface. These blebs appear to contain selected cytosolic proteins [73].

A similar mechanism of protein release via exocytosis has recently been described [74] for a 14.5 KDa lectin (L-14), which is expressed in a wide range of vertebrate tissues. Sequencing of cDNAs including the entire coding length for L-14 from a variety of vertebrates has revealed

no recognizable secretory signal. Following differentiation of myoblasts into myotubes, cytosolic L-14 appears to be concentrated in the cortical cytoplasm (ectoplasm) close to the plasma membrane. The protein then becomes concentrated in restricted regions of the ectoplasm, which progressively evaginate in protrusions of the plasma membrane. These eventually pinch-off and form lectin-rich extracellular vesicles. Some extracellular vesicles appear to be sufficiently permeable to allow immunostaining of L-14 with no prior detergent permeabilization; the final step of L-14 release into the culture medium probably requires disintegration of the extracellular vesicles. Interestingly, the lectin appears to be highly concentrated in some, but not all, of the extracellular vesicles, indicating a possible selective accumulation. Externalization of L-14 is a developmentally regulated process. In undifferentiated myoblasts the lectin is localized in the cytosol, whereas little or no L-14 can be visualized in the cytoplasm of differentiated myotubes. At this stage of differentiation L-14 codistributes with laminin in the extracellular matrix. The externalization of L-14 is similar in many aspects to other developmentally regulated processes. During mineralization of calcifying tissues membrane-bound extracellular structures (matrix vesicles) are formed [75]. These vesicles are enriched in protein originally localized in the cytosol [76]. During erythrocyte maturation, transferrin receptors are selectively internalized into multivesicular bodies, which are then shed by blebbing from the plasma membrane [77]. Basic FGF and L-14 show interesting similarities: They have similar molecular weights; both lack a secretory signal sequence; and they are both found associated with the extracellular matrix. Most important, it is worth recalling that bFGF, like L-14, is involved in several developmental and differentiation processes, including neurite outgrowth [8,9], limb regeneration in amphibians [13–15], mesenchyme differentiation in the Xenopus embryo [10–12], and capillary formation (angiogenesis) [19,20]. In the light of these findings, it appears that selective protein externalization via exocytosis is not an uncommon phenomenon during development and differentiation [78]. Interleukin-1 release during macrophage activation, shedding of transferrin receptors during erythrocyte maturation, and externalization of L-14 during myotube formation are notable examples. Can exocytosis be a route also for bFGF release?

It is not easy to answer this question with conventional experimental methods. Monitoring bFGF release in cell cultures is difficult, or even impossible, because the amounts of growth factor that can be detected in culture fluids are extremely small. More important, under mass culture conditions contamination by bFGF derived from damaged or dead cells cannot be ruled out. In an attempt to overcome this problem, we have employed the phagokinetic track assay described above [66]. Since we had shown that migration of bFGF-transfected NIH 3T3 cells is modulated by the amount of bFGF released from the cells, we reasoned that drugs or treatments that are known to affect the various pathways of protein externalization should also affect migration of our bFGF transfectants in the phagokinetic track assay. If a given treatment resulted in decreased cell motility because of inhibition of bFGF release, then addition of exogenous bFGF should restore normal cell migration. Vice versa, if a particular drug or treatment enhanced cell motility through an increased externalization of bFGF, then neutralizing antibody to bFGF should restore normal cell migration. Drugs or treatments that have an inhibitory or stimulatory effect on cell motility independent of bFGF could thus be distinguished from treatments that affect bFGF release. The results we obtained (Mignatti et al., manuscript submitted for publication) showed that migration of bFGF-transfected NIH 3T3 cells [67] was inhibited by methylamine, serum-free, or low-temperature (18°C), conditions known to block endo- and exocytosis [69,70]. Addition of recombinant bFGF reversed the inhibitory effect of these treatments. The calcium ionophore A23187 [71], which stimulates calcium-dependent exocytosis, dramatically increased the motility of the bFGF transfectants. This treatment had no effect on the control NIH 3T3 cells transfected with the viral vector alone, which produce undetectable amounts of bFGF. The stimulatory effect of A23187 on the motility of the bFGF transfectants was comparable to that obtained by the addition of 1 ng/ml of exogenous bFGF and was reversed by neutralizing anti-bFGF antibody. In contrast, monensin and brefeldin A, which are known to block protein translocation within the ER-Golgi complex [79,80], as well as cyclosporine A, verapamil, and reserpine, which inhibit multidrug resis-

tance (MDR) proteins [81,82], showed no effect on cell motility. The drugs or treatments that significantly affected bFGF release have a relatively nonspecific effect on various cell compartments. Methylamine, serum-free, and low-temperature conditions inhibit endo- and exocytosis, but they may also affect secretion via the ER-Golgi system or through non-vesicular routes. Similarly, A23187 stimulates calcium-dependent exocytosis, but may affect the other two pathways as well. However, monensin and brefeldin A, which only affect protein transport within the ER-Golgi complex, did not inhibit bFGF release. Similarly, cyclosporine A, verapamil, and reserpine, which are substrates for MDR proteins and compete with other molecules exported from the cell via these integral membrane proteins, had no effect on bFGF release. Therefore, the inhibition of bFGF release obtained with methylamine and under serum-free and low-temperature conditions is probably due to inhibition of exocytosis. Thus, in an indirect way, these results show that bFGF release may be achieved through a process of exocytosis similar to those described for IL-1 and L-14. This novel, alternative secretory pathway may represent a general mechanism for protein release from the cells, also used by other proteins with a defined extracellular function but devoid of a secretory signal sequence, such as transglutaminase (blood coagulation factor XIIIa) [83], PD-ECGF [50], ADF, a thioredoxin-like protein released by leukemic cells [84], CNTF [51], thymosine [85], and parathymosine [86].

The intimate molecular mechanism(s) by which bFGF, as well as IL-1 and L-14, can be selectively recognized among other cytosolic proteins and accumulated into secretory vesicles remain(s) unknown. Post-translational modification may provide a signal for selective export. In addition, a possible role for heat-shock and MDR proteins is also worth considering. In yeast, secretion of a-factor, a mating pheromone, requires the presence of the STE 6 gene product, a protein homologous to the mammalian MDR glycoprotein [87]. The transferrin receptor-rich vesicular elements shed during erythrocyte maturation also appear to contain abundant heat-shock protein 70 (hsp 70) [88]. In our experiments, cyclosporine A, verapamil, and reserpine, which inhibit MDR proteins, did not inhibit bFGF release. However, we cannot rule out that MDR proteins other than the ones inhibited by these drugs may contribute to bFGF translocation across the plasma membrane.

In conclusion, a substantial body of experimental evidence shows that bFGF acts extracellularly, although it cannot be secreted via the "classic" secretory pathway. At least three routes may be envisaged for bFGF externalization: cell lysis, sublethal cell injury, and exocytosis. While externalization of cytosolic proteins following cell death may represent a "rescue" mechanism under conditions in which tissue damage occurs (e.g., wound healing and ischemic conditions), exocytosis may be a physiologically relevant route also shared by other proteins. The occurrence of protein release via exocytosis during developmentally regulated processes, such as myotube formation, or during differentiation, as in macrophage activation and erythrocyte maturation, is particularly interesting in light of the involvement of bFGF in embryonic development and in a number of differrentiative processes.

It should be borne in mind, however, that most of our knowledge in this field derives from in vitro studies and thus may be affected by "unnatural," if not artifactual, conditions. A variety of simple cell culture manipulations, such as trypsinization, changing the medium, exposure to serum components (α2-macroglobulin), or temperature shift, have been reported to induce membrane blebbing and stimulate protein release from some cells [89,90]. The "leakage" of bFGF caused by "sublethal cell injury" described by McNeil et al. [63] might be mediated by a mechanism of exocytosis triggered by the experimental procedures used. Stressful conditions might mimic physiological signals that are generated in vivo when bFGF release is required. Thus, "leakage," "sublethal cell injury," or exocytosis might refer, with different words, to the same mechanism for the externalization of bFGF.

This mode of protein release may be physiologically important. The utilization of an alternative route for protein export may serve the purpose of avoiding the oxidizing milieu of the ER. Free thiol groups are present in bFGF, PD-ECGF, IL-1, ADF, and factor XIIIa, all of which lack a secretion signal. Passage through the ER lumen might be detrimental to the biological activities of these proteins, although reducing conditions do not seem to affect bFGF activity. Furthermore, it is noteworthy that a number of cytokines, including bFGF, PD-ECGF, IL-1, CNTF, thymosin, and parathymosin, are devoid

of secretory signal sequences. The lack of a hydrophobic signal peptide results in the segregation of a growth factor from its receptor. This may be necessary to prevent autocrine stimulation. The co-localization of a growth factor and its receptor in the ER-Golgi system may result in receptor activation and uncontrolled stimulation. Direction of bFGF into the ER-Golgi pathway by splicing to a functional signal sequence has been shown to result in neoplastic transformation of the cells that express this construct [59]. Similarly, segregation of IL-1 from potential receptors may be required to prevent uncontrolled lymphocyte proliferation. However, it has recently been shown that cell transformation by K-fgf/hst, which has a signal peptide, requires receptor activation at the cell surface [91]. Retention of the growth factor in the ER-Golgi system results in a significant decrease of cell transformation. Interestingly, as is the case for bFGF, transformation induced by the non-secreted form of K-fgf/hst is reversed by anti-K-fgf/hst antibody, indicating that the growth factor can be externalized via an alternative route.

Several lines of evidence indicate that bFGF may be released via a novel secretion pathway independent of the "classic" ER-Golgi route, and not solely through cell death or injury. Investigating the intimate molecular mechanism by which this is achieved deserves further efforts and will be fundamental for a full comprehension of the physiology of this growth factor.

ACKNOWLEDGMENTS

This work was supported by grants from the American Cancer Society and the National Institutes of Health to D.B.R., and from the Italian Association for Cancer Research and the Italian Ministry of Scientific Research and University to P.M. Studies reported from the authors' laboratories were carried-on during P.M.'s tenure at N.Y.U. Medical Center. We thank M. Vassallo and E. Toufexis for their assistance.

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