Metabolism of Receptor-bound and Matrix-bound Basic Fibroblast Growth Factor by Bovine Capillary Endothelial Cells

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Abstract. Bovine capillary endothelial (BCE) cells were incubated at 4°C with 5 ng/ml ^{125}I-basic fibroblast growth factor (bFGF) to equilibrate ^{125}I-bFGF with high affinity cell surface receptors and low affinity matrix binding sites. 67% of the added ^{125}I-bFGF bound to the matrix and 7% bound to receptors. The fate of bound bFGF was followed after cells were incubated in bFGF-free medium and were shifted to 37°C to restore cell metabolism. ^{125}I-bFGF bound to receptors decreased rapidly while the amount of ^{125}I-bFGF bound to matrix was reduced more slowly. The rapid decrease in receptor-bound ^{125}I-bFGF appeared to be due to a down-regulation of bFGF receptors; cells that had been treated for 5 h with bFGF had 60% fewer high affinity receptors than untreated cells. Despite the initial high level of ^{125}I-bFGF binding to matrix, most of this ^{125}I-bFGF was mobilized and metabolized by the cells. ^{125}I-bFGF was internalized by the cells at 37°C, leading to a constant accumulation of ^{125}I-bFGF within the cell. Internalized bFGF was rapidly cleaved from an 18-kD form to a 16-kD form. The 16-kD form was more slowly degraded with a half-life of ~8 h. Degradation of internalized ^{125}I-bFGF was inhibited by chloroquine, suggesting that the digestion occurred in a lysosomal compartment.

The role of matrix binding sites in the internalization process was investigated. Binding to matrix sites seemed not to be directly involved in the internalization process, since addition of heparin at a concentration that blocked 95% of the binding to matrix had no effect on the initial rate of internalization of bFGF.

BCE cells also released a substance that competed for the binding of bFGF to matrix but not to receptors. This substance bound to DEAE-cellulose and was sensitive to heparinase treatment, suggesting that it was a heparinlike molecule. Thus, heparinlike molecules produced by BCE cells can modulate the cellular interaction with bFGF. Matrix-associated heparinlike molecules bind bFGF which can later be metabolized by the cell, and secreted heparinlike molecules release bFGF from matrices.

During angiogenesis new blood vessels arise from existing microvessels in response to soluble factors (Folkman, 1984). Recently several angiogenesis factors have been purified and have been identified as either acidic or basic fibroblast growth factor (aFGF or bFGF)[1] (Shing et al., 1985; Esch et al., 1985; Lobb et al., 1985; Gospodarowicz et al., 1985; Thomas et al., 1985; Moscatelli et al., 1986*b*; Presta et al., 1986). Presumably the FGFs exert their effects by interaction with high affinity cell surface receptors on the endothelial cells of the microvessels. In binding experiments carried out at 4°C, two classes of binding sites with different affinities for bFGF have been identified on a variety of cultured cells including bovine capillary endothelial (BCE) cells (Neufeld and Gospodarowicz, 1985; Olwin and Hauschka, 1986; Moenner et al., 1986; Moscatelli, 1987). The low affinity binding appears to be due to cell-associated heparinlike molecules since it can be competed with heparin or heparan sulfate, bFGF bound to low affinity sites can be removed with 2 M NaCl at neutral pH (conditions which elute bFGF from heparin affinity columns), and the low affinity binding can be substantially reduced by treating the cells with heparinase (Moscatelli, 1987). High affinity binding is probably due to cell surface receptors for bFGF, and bFGF bound to high affinity sites can be eluted from the cell with 2 M NaCl at pH 4.0 (Moscatelli, 1987). High affinity binding appears to involve binding to two species of receptor with molecular masses of 125 and 145 kD (Neufeld and Gospodarowicz, 1985; Moenner et al., 1986).

High affinity receptors for aFGF have also been identified on a variety of cells (Schreiber et al., 1985; Friesel et al., 1986; Huang et al., 1986). The receptors for aFGF have molecular masses similar to the receptors for bFGF (Friesel et al., 1986; Neufeld and Gospodarowicz, 1986), and aFGF

1. *Abbreviations used in this paper*: aFGF, acidic fibroblast growth factor; BCE cell, bovine capillary endothelial cell; bFGF, basic fibroblast growth factor.

has been reported to compete for the binding of bFGF to its receptor (Neufeld and Gospodarowicz, 1986; Olwin and Hauschka, 1986). The relationship between aFGF and bFGF receptors is presently unclear.

For many hormones and growth factors, interaction with a cell surface receptor at 37°C leads to a rapid internalization of the ligand–receptor complex accompanied by a down-regulation of the number of surface receptors (Carpenter and Cohen, 1976; Heldin et al., 1982; Massague, 1983). The internalized ligand–receptor complex is dissociated in endosomes and the ligand is transferred to lysosomes where it is degraded (Brown et al., 1983). In accordance with this model, the exposure of baby hamster kidney (BHK) cells to bFGF and exposure of mouse lung capillary endothelial cells to aFGF leads to the down-regulation of FGF receptors on these cells (Neufeld and Gospodarowicz, 1985; Friesel et al., 1986). However, it has been reported that there is no down-regulation of bFGF receptors on 3T3 cells (Olwin and Hauschka, 1986).

The role of low affinity binding of bFGF and its relationship to high affinity receptors is unclear. When the binding of bFGF to cell-associated heparinlike molecules is blocked by adding an excess of heparin to the medium, there is no effect on the stimulation of plasminogen activator production by BCE cells (Moscatelli, 1987), implying that the low affinity sites have no direct role in the stimulation of the cell. It has been proposed that the low affinity binding may act as a reservoir of bFGF for the cell, prolonging the response of a single exposure to bFGF (Moscatelli, 1987). In the experiments presented here, the dynamics of bFGF metabolism by BCE cells at 37°C and the role that low affinity binding sites play in this process are investigated.

It is not clear whether the heparinlike molecules involved in low affinity binding are heparan sulfate proteoglycans in the subcellular matrix, heparinlike components of the plasma membrane, or both. For the purposes of this discussion, the term "matrix" will refer to all of these possibilities.

Materials and Methods

Cell Culture

BCE cells were obtained from the adrenal cortex of recently slaughtered cattle and grown under conditions previously described (Folkman et al., 1979; Gross et al., 1982).

Labeling of bFGF

bFGF was purified from term human placentas as previously described (Moscatelli et al., 1986*b*; Presta et al., 1986). Purified bFGF was labeled with ^{125}I by the Iodo-Gen (Pierce Chemical Co., Rockford, IL) procedure as previously described (Neufeld and Gospodarowicz, 1985; Moscatelli, 1987). ^{125}I-bFGF used in these experiments had a specific activity of ~1,000 cpm/fmol.

Determination of Binding Constants for BCE Cells

Confluent cultures of BCE cells on 60-mm dishes were washed three times with cold PBS and were incubated on an orbital shaker at 4°C with cold αMEM containing 0.15% (wt/vol) gelatin, 25 mM Hepes, pH 7.5, and various concentrations of ^{125}I-bFGF. After 2 h, the medium was removed and the cells were washed three times with PBS, twice with 1 ml 2 M NaCl in 20 mM Hepes, pH 7.5, and twice with 1 ml 2 M NaCl in 20 mM sodium acetate, pH 4.0. The radioactivity released by the acid 2-M NaCl wash, representing ^{125}I-bFGF bound to high affinity sites (Moscatelli, 1987), was analyzed by the method of Scatchard (1949).

^{125}I-bFGF Internalization Experiments

Confluent cultures of BCE cells on 60-mm dishes (1.2–1.5 × 10^6 cells) were washed three times with cold PBS and were incubated at 4°C on an orbital shaker in cold αMEM containing 0.15% (wt/vol) gelatin, 25 mM Hepes, pH 7.5, and 5 ng/ml ^{125}I-bFGF. After a 2-h incubation period, which allowed the ^{125}I-bFGF to reach equilibrium with cellular binding sites, the medium was removed, cultures were washed three times with cold PBS, and the medium was replaced with αMEM containing 0.15% gelatin and 25 mM Hepes, pH 7.5, at the appropriate temperature. Half of the cultures were incubated further at 4°C and half were transferred to an orbital shaker at 37°C. At various times after the change of medium, the medium was removed; the cells were washed three times with PBS; twice with 1 ml 2 M NaCl in 20 mM Hepes, pH 7.5; twice with 1 ml 2 M NaCl in 20 mM sodium acetate, pH 4.0; and were extracted with 1 ml 0.5% Triton X-100 in 0.1 M sodium phosphate, pH 8.1. Radioactivity in the medium, the combined neutral 2 M NaCl washes, the combined acid 2 M NaCl washes, and the Triton X-100 extracts was determined with a gamma scintillation counter (model 5210, Packard Instrument Co., Inc., Downers Grove, IL).

Down-Regulation of bFGF Receptors

Confluent cultures of BCE cells were incubated for 5 h at 37°C with αMEM containing 0.15% gelatin and either 5 ng/ml bFGF or no bFGF. Cells incubated with 5 ng/ml bFGF and half of the cells incubated in medium without bFGF were washed twice with cold 2 M NaCl in 20 mM sodium acetate, pH 4.0, to remove bound bFGF, and then washed three times with cold PBS. The remaining cells that had been incubated in medium without bFGF were just washed three times with cold PBS. These cells were then incubated for 2 h at 4°C with various concentrations of ^{125}I-bFGF and were assayed for high affinity binding sites as described above.

SDS-PAGE of Internalized bFGF

BCE cells were preincubated with 5 ng/ml ^{125}I-bFGF for 2 h at 4°C as described above. The cells were transferred to an orbital shaker at 37°C to initiate internalization. After 30 min, the medium was removed, the cells were washed three times with cold 2 M NaCl in 20 mM sodium acetate, pH 4.0, to remove surface bound ^{125}I-bFGF, and then three times with cold PBS. The medium was replaced with warm bFGF-free αMEM containing 0.15% gelatin and 25 mM Hepes, pH 7.5, and the cells were returned to the orbital shaker at 37°C. At various times thereafter the cells were washed three times with cold PBS and three times with 2 M NaCl in 20 mM sodium acetate, pH 4.0. The cells were scraped from the dish with a rubber policeman and were suspended in 0.1 ml 0.5% Triton X-100 in 0.1 M sodium phosphate, pH 8.1. An equal volume of twofold concentrated electrophoresis sample buffer was added, and the mixture was immediately heated in a boiling water bath for 5 min. The samples were run under reducing conditions on SDS-PAGE with 15% polyacrylamide resolving gels (Laemmli, 1970). The gels were dried and exposed to Kodak (Eastman Kodak Co., Rochester, NY) X-omat AR film. Cells washed with the same procedure and then incubated in medium containing ^{125}I-bFGF continued to accumulate radioactivity inside the cell at about the same rate as unwashed cells, demonstrating that the wash procedure did not harm the cells.

Competition for Binding with Conditioned Medium

Confluent cultures of BCE cells on 60-mm dishes were washed twice with PBS and were incubated at 37°C in αMEM containing 0.15% gelatin and 25 mM Hepes, pH 7.5. At various times, the medium was collected from two of the dishes, centrifuged at 200 *g* for 10 min, and stored at 4°C. After the 24-h time point was collected, ^{125}I-bFGF was added to all the conditioned media to a final concentration of 5 ng/ml. The conditioned media were then incubated on sister cultures of BCE cells on 60-mm dishes for 2 h at 4°C. ^{125}I-bFGF bound to low and high affinity sites on these cells was determined as described above.

Heparinase Treatment and DEAE–Cellulose Chromatography of Conditioned Medium

Confluent cultures of BCE cells on 60-mm dishes were incubated in serum-free αMEM for 24 h at 37°C. The medium was collected and centrifuged at 200 *g* for 5 min. 5-ml aliquots of the conditioned medium were incubated for 6 h at 37°C with or without 10 U/ml of *Flavobacterium heparinum* heparinase (Sigma Chemical Co., St. Louis, MO). The reaction was

stopped by heating the samples for 5 min in a boiling water bath. These samples were then passed through 1-ml DEAE-cellulose (DE 52; Whatman Laboratory Products Inc., Clifton, NJ) columns equilibrated with PBS. The columns were washed with PBS and bound material was eluted with 0.5 M NaCl in 25 mM Hepes, pH 7.5. Ten 0.3-ml fractions were collected from each column. Samples of the original conditioned medium, of the nonbound fractions, and of the elution fractions were assayed for their ability to compete for binding of ^{125}I-bFGF to low affinity sites. The elution fractions were diluted with water so that the final NaCl concentration was 0.15 M. As a control, a sample of the elution buffer was diluted to the same extent and was used in the binding assay. ^{125}I-bFGF and gelatin were added to 1 ml samples of fresh αMEM, of conditioned medium, of the nonbound fractions, or of the diluted elution fractions to achieve final concentrations of 5 ng/ml and 0.15%, respectively. These samples were then incubated on BCE cells in the 16-mm wells of a 24-well plate for 2 h at 4°C. ^{125}I-bFGF bound to low affinity sites on these cells was determined as described above.

Results

Fate of Bound bFGF at 37°C

BCE cells were incubated with 5 ng/ml ^{125}I-bFGF for 2 h at 4°C to prebind bFGF to cellular binding sites. The cells were then washed with PBS, the medium was replaced with bFGF-free medium, and the cells were transferred to 37°C or maintained at 4°C. The fate of the bound bFGF was followed for the next 24 h in four compartments: bFGF in the culture medium; cell-bound ^{125}I-bFGF eluted with 2 M NaCl at neutral pH, which represents bFGF bound to low affinity sites in the matrix; cell-bound ^{125}I-bFGF eluted with a subsequent wash with 2 M NaCl at pH 4.0, which represents bFGF bound to high affinity receptors; and ^{125}I-bFGF released by a subsequent extraction with 0.5% Triton X-100, which represents internalized bFGF.

When the original medium was removed and replaced with bFGF-free medium, 74% of the ^{125}I-bFGF originally added to the cultures was in a cell-associated form, and thus remained with the cultures (67% on low affinity matrix sites and 7% on high affinity receptors). When the cells were incubated in bFGF-free medium at 37°C, there was a rapid decrease in ^{125}I-bFGF bound to high affinity sites (Fig. 1 *A*, •) with about half of the receptor-bound ^{125}I-bFGF disappearing in the first half hour. By 8 and 24 h after transfer, ^{125}I-bFGF bound to receptors was decreased 82%. This decrease was dependent on cell metabolism, since no change in receptor-bound ^{125}I-bFGF occurred at 4°C (Fig. 1 *A*, ○).

After the shift to 37°C, ^{125}I-bFGF bound to matrix decreased continuously (Fig. 1 *B*, •). ^{125}I-bFGF in this fraction was depleted by ~50% by 1 h, but decreased more slowly after that. A decrease in matrix-bound ^{125}I-bFGF was also seen at 4°C. When cells were incubated in FGF-free medium at 4°C, 20% of the matrix-bound ^{125}I-bFGF was lost from this fraction (Fig. 1 *B*, ○) and appeared in the medium (Fig. 1 *C*, ○). Thus, part of the decrease in matrix-bound ^{125}I-bFGF at 37°C was probably due to a similar redistribution of ^{125}I-bFGF between bound and free states. At 37°C only 4% of the ^{125}I-bFGF that was originally matrix-associated remained matrix-bound by 8 h. The ^{125}I-bFGF released into the medium at 37°C (Fig. 1 *C*, •) can account for only 35% of the ^{125}I-bFGF originally bound to the matrix. It is likely that the remainder of the matrix-bound ^{125}I-bFGF was internalized by the cells. These results indicate that most of the matrix-bound bFGF was freely available for metabolism by the cell. The mechanism by which matrix-bound ^{125}I-bFGF was internalized is unclear.

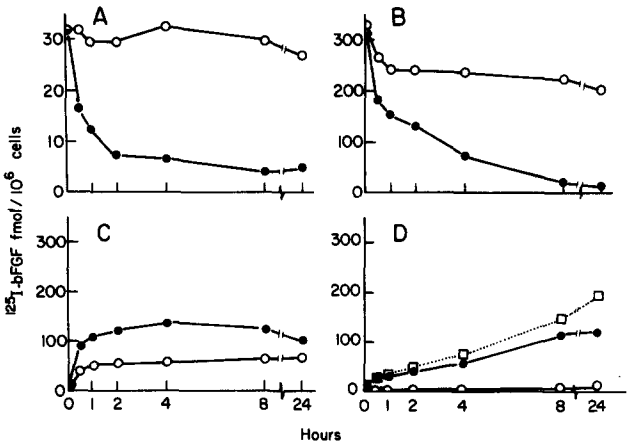

Figure 1. Fate of ^{125}I-bFGF in various BCE cell fractions. Confluent cultures of BCE cells on 60-nm dishes were incubated for 2 h at 4°C with αMEM containing 0.15% gelatin, 25 mM Hepes, pH 7.5, and 5 ng/ml ^{125}I-bFGF. Then the medium was removed, the cells were washed once with PBS, and the medium was replaced with αMEM containing only gelatin and Hepes. Half of these cells were incubated further at 4°C (○) and half were incubated at 37°C (•). At the indicated times, the medium was removed and assayed for TCA-precipitable radioactivity (*C*). The cells were washed three times with PBS, twice with 2 M NaCl at pH 7.5 (*B*), and twice with 2 M NaCl at pH 4.0 (*A*). The cells were then extracted with 0.5% Triton X-100 (*D*). The amount of radioactivity released in each of the washes and in the extract was determined. In addition, the amount of TCA-soluble radioactivity detected in the medium was added to the amount of radioactivity in the cell extracts (□). Each point represents the average of results obtained with duplicate cultures. The SD for each point was ≤8%. The experiment was performed three times with similar results.

^{125}I-bFGF accumulated within the cell at an approximately constant rate in the first 8 h after transfer to 37°C (Fig. 1 *D*, •) as seen by an increase in radioactivity that was not removed by washing the cells with 2 M NaCl, at pH 7.5 or pH 4.0, and that could be recovered with a subsequent extraction with 0.5% Triton X-100. No increase in this fraction was observed in the absence of cell metabolism at 4°C (Fig. 1 *D*, ○). Over 90% of the internalized bFGF remained in a TCA-precipitable form in the first 4 h after the shift to 37°C (data not shown). At 24 h, BCE cells contained the same amount of ^{125}I-bFGF as at 8 h, and 85% of this internalized ^{125}I-bFGF was still TCA precipitable.

The amount of ^{125}I-bFGF in the medium increased abruptly in the first half hour after transfer to 37°C (Fig. 1 *C*, •). All of the radioactivity in the medium was TCA precipitable in the first hour at 37°C. However, at 2 h and later, a small percentage of the radioactivity in the medium was in a TCA-soluble form. When this experiment was performed on cells incubated in 50 μM chloroquine, an inhibitor of lysosomal degradation, no TCA-soluble radioactivity appeared in the medium (data not shown), suggesting that the TCA-soluble radioactivity represented internalized ^{125}I-bFGF that was degraded by the cell and subsequently released. The amount of TCA-soluble radioactivity was added to the amount of ^{125}I-bFGF accumulated by the cells (Fig. 1 *D*, □) to give a better estimate of the rate of internalization. It appeared that ^{125}I-bFGF had been internalized at an approximately

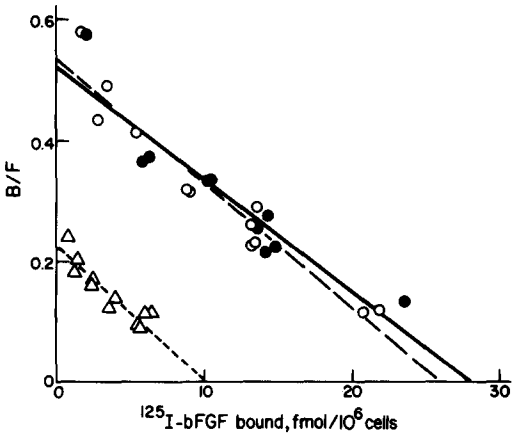

Figure 2. Down-regulation of high affinity bFGF binding sites. Confluent cultures of BCE cells on 60-mm dishes were incubated for 5 h at 37°C in αMEM containing 0.15% gelatin and either 5 ng/ml bFGF or no bFGF. The cells incubated with bFGF (△) and half the cells incubated without bFGF (○) were washed three times with 2 M NaCl at pH 4.0 and three times with PBS. The remainder of the cells incubated without bFGF (●) were only washed three times with PBS. The cells were then used to assay high affinity binding sites as described in Materials and Methods. Scatchard analysis of the high affinity binding data is shown here.

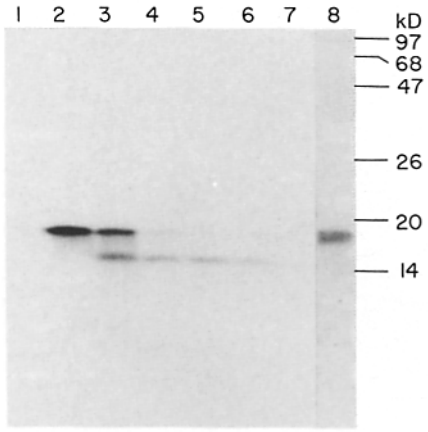

Figure 3. Metabolism of internalized bFGF. Confluent cultures of BCE cells on 60-mm dishes were incubated for 2 h at 4°C in αMEM containing 0.15% gelatin, 25 mM Hepes, pH 7.5, and 5 ng/ml ^{125}I-bFGF. The cells were transferred to 37°C. After 30 min, the cells were washed three times with 2 M NaCl at pH 4.0 and three times with PBS, the medium was replaced with bFGF-free medium, and the cells were incubated further at 37°C. At various times the cells were washed three times with PBS and three times with 2 M NaCl at pH 4.0. The cells were extracted and the extracts were run on a 15% polyacrylamide gel under reducing conditions. Lane *1*, after preincubation at 4°C; lane *2*, after 30 min at 37°C; lanes *3–7*, 30 min at 37°C followed by stripping of bound ^{125}I-bFGF, and incubation in bFGF-free medium for 0.5, 1.5, 3.5, 7.5, or 24 h at 37°C; lane *8*, cells preincubated for 2 h at 37°C with 50 μM chloroquine and then treated as in lane *4*.

constant rate over the first 8 h at 37°C and continued to be internalized afterward.

Down-Regulation of High Affinity Receptors

The rapid decrease in ^{125}I-bFGF bound to receptors after BCE cells with prebound ^{125}I-bFGF were transferred to 37°C suggested that the receptors were down-regulated. To test if down-regulation occurs in these cells, BCE cells were incubated with 5 ng/ml unlabeled bFGF for 5 h at 37°C. These cells were then washed with 2 M NaCl at pH 4.0 to remove bound bFGF and were used for binding studies at 4°C. Scatchard analysis of the binding data revealed that the bFGF-treated cells had 60% fewer receptors than untreated cells (Fig. 2). Untreated cells washed with 2 M NaCl at pH 4.0 had the same number of high affinity binding sites as untreated cells washed with PBS, demonstrating that the wash procedure had no effect on the receptors (Fig. 2). None of these treatments affected the affinity of the receptors for bFGF as shown by the fact that the slopes of the Scatchard plots were equal (Fig. 2). From the Scatchard analysis, it was calculated that cells preincubated with bFGF had 6,000 receptors/cell with a K_d of 2.2 × 10^{-11} M, untreated cells washed with PBS had 16,900 receptors/cell with a K_d of 2.7 × 10^{-11} M, and untreated cells washed with 2 M NaCl at pH 4.0 had 15,500 receptors/cell with a K_d of 2.4 × 10^{-11} M. Thus, exposure to bFGF caused down-regulation of bFGF receptors on BCE cells.

The BCE cells treated with 5 ng/ml bFGF for 5 h at 37°C bound the same amount of ^{125}I-bFGF to matrix sites as untreated cells (data not shown). The number of low affinity binding sites in the matrix was apparently not altered by exposure to bFGF.

Internalized bFGF

The fate of internalized ^{125}I-bFGF was followed by autoradiography. BCE cells were preincubated with ^{125}I-bFGF for 2 h at 4°C, warmed to 37°C for 30 min to initiate internalization, washed with 2 M NaCl at pH 4.0 to remove surface-bound ^{125}I-bFGF, and then incubated at 37°C in bFGF-free medium. In these cells, internalized radioactivity decreased with a half-life of ~8 h (data not shown). Extracts of the cells made at various times after initiation of internalization were run on SDS-PAGE and the internalized ^{125}I-bFGF was visualized by autoradiography. The ^{125}I-bFGF internalized in the first half hour remained mostly in its native 18-kD form (Fig. 3). After 2 h, most of the 18-kD bFGF was converted to a 16-kD form. Subsequently, the internalized ^{125}I-bFGF was slowly degraded to smaller forms, but some persisted in the 16-kD form for 24 h (Fig. 3). In cells that had been treated with 50 μM chloroquine, the internalized ^{125}I-bFGF was not degraded (Fig. 3, lane *8*), suggesting that the degradation occurs through a lysosomal pathway.

Effect of Heparin on Internalization of bFGF

To test whether bFGF binding to cell-associated heparinlike molecules has an integral role in the internalization of bFGF by the cells, the ability of BCE cells to internalize ^{125}I-bFGF was studied under conditions in which the binding of ^{125}I-bFGF to matrix was blocked. Addition of heparin to the medium can block the binding of ^{125}I-bFGF to matrix on BHK cells and BCE cells (Moscatelli, 1987). The ability of BCE cells to internalize ^{125}I-bFGF in the presence of 10 μg/ml heparin was investigated.

Addition of 10 μg/ml heparin blocked >95% of the ^{125}I-bFGF binding to BCE cell matrix (Fig. 4 *B*). However, the amount of ^{125}I-bFGF bound to high affinity receptors

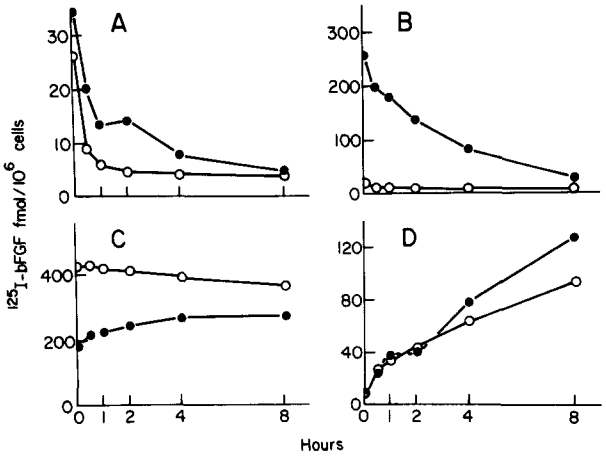

Figure 4. Fate of ^{125}I-bFGF in various BCE cell fractions in the presence of heparin. Confluent cultures of BCE cells on 60-mm dishes were incubated for 2 h at 4°C with αMEM containing 0.15% gelatin, 25 mM Hepes (pH 7.5), and 5 ng/ml ^{125}I-bFGF, with (○) or without (●) 10 μg/ml heparin. The cells were then transferred to 37°C. At the indicated times, the medium was removed and assayed for TCA-precipitable radioactivity (*C*). The cells were washed three times with PBS, twice with 2 M NaCl at pH 7.5 (*B*), and twice with 2 M NaCl at pH 4.0 (*A*). The cells were then extracted with 0.5% Triton X-100 (*D*). The amount of radioactivity in each of the washes and in the extract was determined. Each point represents the average of results obtained with duplicate cultures. SDs for each point were ≤8%. The experiment was performed twice with similar results.

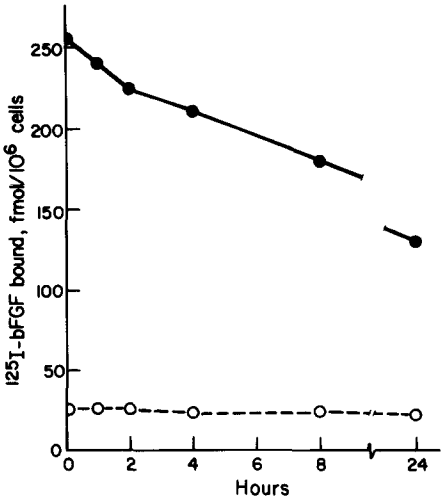

Figure 5. Competition by conditioned medium for ^{125}I-bFGF binding to BCE cells. Confluent cultures of BCE cells were incubated at 37°C in αMEM containing 0.15% gelatin and 25 mM Hepes (pH 7.5). At the indicated times, the medium was removed and stored at 4°C. ^{125}I-bFGF was added to the conditioned medium to achieve a final concentration of 5 ng/ml and this medium was incubated on confluent sister cultures of BCE cells at 4°C. After a 2-h incubation period, high (○) and low (●) affinity binding was determined as described in Materials and Methods.

was only slightly lower in BCE cells exposed to heparin than in control cells (Fig. 4 *A*). Upon transfer to 37°C, ^{125}I-bFGF bound to receptors decreased rapidly both in control cells and in cells exposed to heparin, resulting in an 80% reduction by 4 h (Fig. 4 *A*). In the first 2 h after transfer to 37°C, there was a twofold decrease both in ^{125}I-bFGF bound to matrix in control cells and in the residual ^{125}I-bFGF bound to matrix on cells exposed to heparin (Fig. 4 *B*). After 2 h, there was a further decrease in ^{125}I-bFGF bound to matrix on control cells but not on cells exposed to heparin. Despite the dramatic difference in the amount of ^{125}I-bFGF bound to matrix, in the first 2 h after transfer to 37°C BCE cells internalized ^{125}I-bFGF at the same rate in the presence and absence of heparin (Fig. 4 *D*). After 4 and 8 h, slightly less ^{125}I-bFGF was internalized by cells exposed to heparin (Fig. 4 *D*). Thus, the ability of ^{125}I-bFGF to bind to cell-associated heparinlike molecules had little effect on its initial rate of internalization by the cell.

Release of a Competitor for Low Affinity Binding

In cultures of BCE cells that had been preincubated with ^{125}I-bFGF at 4°C and then transferred to 37°C, there was a continuous decrease in ^{125}I-bFGF bound to matrix (Fig. 1 *B*). The decrease in binding was not due to a decrease in the number of matrix binding sites (see above). Part of the decrease could be explained by the internalization of ^{125}I-bFGF that was originally bound to matrix. However, part of the matrix-bound ^{125}I-bFGF was released into the medium (Fig. 1 *C*) and this released bFGF seemed less capable of rebinding to the matrix. These results suggested that BCE cells might be secreting a molecule which competes for the matrix binding thereby preventing the ^{125}I-bFGF in the medium from binding to heparinlike molecules on the cell.

To test whether BCE cells were secreting such a molecule, the cells were incubated at 37°C in serum-free medium for various periods, and the medium was collected and used for binding assays at 4°C. The conditioned medium from BCE cells partially competed for ^{125}I-bFGF binding to matrix but not for binding to receptors (Fig. 5). The extent of competition depended on the length of the conditioning period. Thus, BCE cells at 37°C appear to release into the medium a molecule which competes with the binding of bFGF to the matrix. The competitor could be depleted from the conditioned medium by passing the medium over a DEAE-cellulose column (Table I). The competitor was concentrated on

Table I. Effect of DEAE-Cellulose Chromatography and Heparinase Treatment on the Competitor in Conditioned Medium

	^{125}I-bFGF bound to matrix
	(fmol/10^6 cells)
Fresh medium	215 ± 2
Conditioned medium	132 ± 2
DEAE-cellulose flow through	228 ± 5
DEAE-cellulose eluate	65 ± 5
DEAE-cellulose eluate (heparinase pretreated)	176 ± 11

Confluent cultures of BCE cells were incubated 24 h in serum-free medium. The conditioned medium was collected and aliquots were incubated for 6 h at 37°C with or without the addition of 10 U/ml *Flavobacterium heparinum* heparinase. The treated conditioned media were then applied to DEAE-cellulose columns. The columns were washed with PBS and eluted with 0.5 M NaCl in 25 mM Hepes, pH 7.5. The effect of various fractions on binding of ^{125}I-bFGF to low affinity matrix sites was compared. ^{125}I-bFGF was added to the samples to achieve a final concentration of 5 ng/ml and these samples were incubated on confluent cultures of BCE cells at 4°C. After a 2-h incubation period, low affinity binding was determined as described in Materials and Methods.

the column and was eluted with 0.5 M NaCl (Table I). Treatment of the conditioned medium with heparinase diminished the inhibitory capacity of the DEAE-cellulose eluate (Table I). These results suggest that the competitor is a heparinlike molecule. This molecule has been further characterized as a heparan sulfate proteoglycan (Saksela et al., 1988).

Discussion

The fate of the ^{125}I-bFGF bound to BCE cells at first appears somewhat complicated due to the presence of two classes of binding sites for bFGF on these cells. As with other polypeptide growth factors (Carpenter and Cohen, 1976; Heldin et al., 1982; Massague, 1983), there was a rapid internalization of bFGF causing a down-regulation of the number of high affinity receptors on the cell. FGF receptors previously have been reported to be down-regulated on BHK cells (Neufeld and Gospodarowicz, 1985) and on mouse lung capillary endothelial cells (Friesel et al., 1986) but not on 3T3 cells (Olwin and Hauschka, 1986). However, in contrast to other growth factors which are rapidly degraded inside the cell (King and Cuatrecasas, 1981; Heldin et al., 1982; Bowen-Pope and Ross, 1982; Massague and Kelly, 1986; Walker and Burgess, 1987), the internalized bFGF accumulated in a TCA-precipitable form in the cell. The internalized bFGF was degraded to a 16-kD form within 2 h after its uptake into the cell. The 16-kD form was degraded more slowly and a portion persisted for ≤24 h. Degradation of internalized bFGF was inhibited by chloroquine, a classical inhibitor of lysosomal function, implying that the degradation occurred through a typical lysosomal pathway. A similar cleavage of 18-kD bFGF to a 16.5-kD form by an acid protease has been observed in extracts of human hepatoma cells (Klagsbrun et al., 1987). Apparently in BCE cells, the rate of degradation of internalized bFGF was slower than its rate of uptake, leading to an accumulation of bFGF within the cell.

A significant aspect of the interaction of bFGF with cultured cells is the high capacity of the matrix to bind bFGF. With >1,000,000 matrix binding sites per cell (Moscatelli, 1987) and only 6,000–17,000 receptors per cell, most of the bFGF added to BCE cell cultures binds to matrix. This can be seen in the experiment of Fig. 1 where after a 2-h preincubation with 5 ng/ml ^{125}I-bFGF at 4°C, ~67% of the bFGF added to the culture was bound to matrix while 7% was bound to receptors. This high level of matrix binding may permit the cells to retain most of their bFGF despite fluctuations in the amount of bFGF in the fluid phase. Thus, when the medium of the BCE cell cultures was replaced with FGF-free medium, only 26% of the bFGF originally added was lost. However, the matrix-bound bFGF seems to be easily mobilized. After an 8-h incubation at 37°C, only 4% of the bFGF originally associated with the matrix remained matrix bound. Thus, despite the extensive binding of exogenous bFGF to the matrix, most of this bound bFGF is still available for interaction with the cells.

Although the matrix-bound bFGF may serve as a source of bFGF which eventually binds to high affinity sites and is internalized by the cell, the mechanism by which this occurs is unclear. One possibility is that there is a direct transfer of bFGF from the cell-associated heparinlike molecules to receptors. Such an interaction would facilitate binding of bFGF to the receptors and, as a consequence, would enhance uptake of bFGF by the cell. However, the matrix binding sites did not seem to have a direct role in the internalization process. Internalization of bFGF proceeded at the same rate in cultures with bFGF bound to matrix and in cultures with 95% of the matrix binding blocked. This suggests that there is no direct coupling between the majority of the cell-associated heparinlike molecules and the high affinity receptors.

As would be expected from its binding to heparin (Shing et al., 1984; Gospodarowicz et al., 1984) and cell-associated heparinlike molecules (Moscatelli, 1987), bFGF has been found in isolated extracellular matrices (Shing et al., 1984; Hauschka et al., 1986; Vlodavsky et al., 1987*a*; Jeanny et al., 1987). It has been proposed that extracellular matrix-bound bFGF represents a storage form of bFGF and that it can be liberated by the action of heparinase-like enzymes in order to stimulate endothelial cell proliferation (Baird and Ling, 1987; Vlodavsky et al., 1987*a*). Alternatively, bFGF may be taken up from these sites by neighboring cells with high affinity receptors for bFGF simply by mass action movement of molecules from sites with lower affinity to sites with higher affinity. When unoccupied receptors appear on the surface of cells, they would bind free bFGF from the fluid phase altering the equilibrium between bound and free bFGF. bFGF would be displaced from the matrix to restore the equilibrium. While the experiments presented here do not distinguish between these two possibilities, they do suggest that matrix-bound bFGF is freely available to high affinity receptors on BCE cells.

The metabolism of bFGF by BCE cells is further complicated by the release from the cells of a competitor of bFGF binding to matrix. The competition could be due either to secretion of a bFGF-like molecule by the cells or to the release of a bFGF-binding molecule. Cultured endothelial cells have been shown to produce bFGF (Moscatelli et al., 1986*a*; Schweigerer et al., 1987; Vlodavsky et al., 1987*b*), but it appears that most of the bFGF produced by endothelial cells remains cell-associated and is not released into the medium. Furthermore, the competition obtained with conditioned medium seems not to be due to endothelial cell–derived bFGF because there was little competition with receptor binding. Unlabeled bFGF secreted by the cells would be expected to compete with receptor binding at lower concentrations than are necessary for competition with matrix binding (Moscatelli, 1987). However, the competition obtained with conditioned medium does resemble the competition obtained with heparin (Moscatelli, 1987), where concentrations that competed for 95% of the binding to low affinity sites had little effect on high affinity binding. The competitor from conditioned medium has been partially purified, and has been biochemically characterized as a heparan sulfate proteoglycan (Saksela et al., 1988).

These released heparan sulfate proteoglycans can remove bFGF from matrix binding sites, and thus present another means of nonenzymatically mobilizing bFGF from storage sites in the extracellular matrix. It has been demonstrated that bFGF bound to heparin is much less sensitive to denaturation (Gospodarowicz and Cheng, 1986). The endothelial cell–derived heparan sulfate proteoglycans have a similar protective effect, preventing degradation of bFGF by proteolytic enzymes (Saksela et al., 1988). In vivo, the released

heparan sulfate proteoglycans may serve as carrier molecules for bFGF, facilitating the distribution of bFGF throughout the body while protecting it from degradation.

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