

# In Vitro Angiogenesis on the Human Amniotic Membrane: Requirement for Basic Fibroblast Growth Factor-induced Proteinases

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**Abstract.** The role of basic fibroblast growth factor (bFGF) induced proteinases in basement membrane (BM) invasion by bovine capillary endothelial (BCE) cells was studied using a quantitative in vitro assay previously described (Mignatti et al., 1986). <sup>125</sup>I-iododeoxyuridine-labeled BCE cells were grown for 72 h on the human amnion BM, and cell invasion was determined by measuring the radioactivity associated with the tissue after removal of the noninvasive cell layer. BCE cells were noninvasive under normal conditions. Addition of human bFGF to either the BM or to the stromal aspect of the amnion induced BCE cell invasion with a dose-dependent response. This effect

was maximal in the presence of 70 ng/ml bFGF, and was inhibited by anti-bFGF antibody. Transforming growth factor beta, as well as plasmin inhibitors and anti-tissue type plasminogen activator antibody inhibited BCE cell invasion. The tissue inhibitor of metalloproteinases, 1-10 phenanthroline, anti-type IV and anti-interstitial collagenase antibodies had the same effect. On the contrary, anti-stromelysin antibody and Eglin, an inhibitor of elastase, were ineffective. The results obtained show that both the plasminogen activator-plasmin system and specific collagenases are involved in the invasive process occurring during angiogenesis.

**T**HE formation of new capillaries occurs in a variety of normal and pathologic conditions, including embryo and organ development, wound healing, and tumor growth. In the past few years a number of normal and tumor cells have been reported to produce polypeptide factors capable of inducing the formation of capillaries either in vitro and/or in vivo. Among the angiogenic factors so far purified and characterized, basic fibroblast growth factor (bFGF)<sup>1</sup> appears to be one of the more potent angiogenesis inducers (Folkman, 1986; Gospodarowicz et al., 1986; Lobb et al., 1986; Schweigerer et al., 1987). The response of microvascular endothelial cells to bFGF in vitro consists of three major components: an increase in the rate of cell proliferation, a stimulation of endothelial cell migration along a gradient of angiogenic factor, and an increased production of proteolytic enzymes such as plasminogen activator (PA) and collagenase (Moscattelli et al., 1986; Presta et al., 1986). The production of proteinases by endothelial cells is believed to be of fundamental importance for the degradation of the perivascular extracellular matrix and of the stroma of the tis-

sue to be vascularized. In this regard microvascular endothelial cells appear to behave similarly to invasive tumor cells in that they are able to cross the anatomical barriers, basement membranes (BM), and interstitial stroma that separate the tissue compartments of the organism (Mullins and Rohrlach, 1983; Danø et al., 1985; Moscatelli and Rifkin, 1988).

It has been proposed that during angiogenesis, as well as in tumor invasion, a cascade of proteolytic events occurs, which leads to the degradation of the extracellular matrix (Gross et al., 1983; Mignatti et al., 1986). The PA produced by endothelial cells converts plasminogen to plasmin, a serine proteinase with trypsin-like specificity. Since the amount of plasminogen present in most tissues is relatively high, the production of even small amounts of PA may result in high levels of local plasmin activity. Moreover, while plasmin is capable of degrading major extracellular matrix components, including fibronectin and laminin (Werb et al., 1980), recent evidence also points to a central role for plasmin in the activation of other enzymes. Although the importance of PAs in matrix degradation has been debated (Kramer et al., 1982; Bogenman and Jones, 1983; Heisel et al., 1983; Bergman et al., 1986), a number of reports indicate that PA may catalyze the degradation of collagen through the formation of plasmin, which acts as an activator of the zymogen forms of collagenases secreted by most cells (Werb et al., 1977;

1. *Abbreviations used in this paper:* BCE, bovine capillary endothelial; bFGF, basic fibroblast growth factor; BM, basement membrane; DOC, Nadeoxycholate; PA, plasminogen activator; TGF  $\beta$ , transforming growth factor beta; tPA, tissue type plasminogen activator; uPA, urokinase plasminogen activator.

Paranjpe et al., 1980; O'Grady et al., 1981; Salo et al., 1982; Gavrilovic et al., 1985). Endothelial cells have been reported to produce interstitial, as well as type IV collagenase (Kalebic et al., 1983; Gross et al., 1983). Type IV collagenase degrades type IV collagen, the basement membrane collagen, and it has been shown that plasmin can activate type IV collagenase (Salo et al., 1982). Thus, the production of a proteinase with restricted specificity, such as PA, can result in the generation of several active proteinases: plasmin, with a broad substrate specificity, and the collagenases, whose spectrum is restricted. This array of proteinases can afford the degradation of and infiltration into the basement membrane by microvascular endothelial cells.

However, while the production of proteinases by endothelial cells in response to angiogenic factors has been documented, the role of enzymes in angiogenesis has not been demonstrated experimentally. This is mainly because of the lack of a simple and biologically relevant *in vitro* model for the capillary wall. The bioassays for angiogenesis used so far, neovascularization of the rabbit cornea (Gimbrone et al., 1974) or angiogenesis on the chick chorioallantoic membrane (Ausprunk et al., 1975), are cumbersome and non-quantitative. Moreover, they have distinct drawbacks since host factors, such as those involved in inflammation, can influence the angiogenic response.

Attempts have been made to develop *in vitro* angiogenesis assays. Rat aortic rings cultured in plasma clot have been reported to give rise to a microvascular network consisting of branching endothelial channels (Nicosia et al., 1983). Montesano et al. (1983) have reported that capillary endothelial cells grown on a three-dimensional type I collagen gel can invade into the culture substrate and form tube-like structures resembling capillaries. This phenomenon has been referred to as "in vitro angiogenesis" and can be induced by treatment with tumor promoters or with bFGF (Montesano and Orci, 1985; Montesano et al., 1986). More recently, fibrin gels have also been used to study microvascular endothelial cell invasion and differentiation into structures resembling capillaries (Montesano et al., 1987). These experimental models provide relatively simple, but nonquantitative, *in vitro* assays for angiogenesis. However, the artificial conditions of the systems used are far from representing the environment in which angiogenesis occurs *in vivo*. The capillary basal lamina mainly consists of BM (type IV) collagen, which is distinct from interstitial collagens and whose degradation has very specific proteolytic requirements (Yurchenco and Ruben, 1987). Moreover, several major glycoproteins, including fibronectin and laminin, as well as heparan sulfate proteoglycans are also present in the BM (Liotta et al., 1983; Madri et al., 1983).

The BM of the human amnion has been used by other workers as a growth substrate for microvascular endothelial cells (Madri et al., 1983; Furie et al., 1984). This model provides experimental conditions that are close to those found *in vivo*. Under these conditions, microvascular endothelial cells differentiate spontaneously into tube-like structures resembling capillaries, although they do not invade through the BM (Madri et al., 1983; Furie et al., 1984). However, angiogenesis inducers were not used in these experiments, and the authors did not report whether the endothelial cells used spontaneously produced any of the proteinases that might promote degradation of and invasion through the BM.

We have recently described a quantitative *in vitro* assay for tumor invasion (Mignatti et al., 1986), which consists in determining the number of <sup>125</sup>I-iododeoxyuridine-labeled cells that infiltrate through the BM and invade into the underlying stroma of the human amnion. We have now used the same experimental system to study invasion of the BM by bovine capillary endothelial (BCE) cells stimulated with bFGF. The effects of synthetic and natural inhibitors of and antibodies to serine- and metalloproteinases were studied to understand the role of these enzymes in the invasive processes that occur during angiogenesis. The results from this *in vitro* study indicate that bFGF can induce microvascular endothelial cells to invade through the amnion BM, and that the molecular mechanisms mediating this process involve a cascade of serine- and metalloproteinase activations similar to that observed for tumor invasion.

## Materials and Methods

### Cells and Media

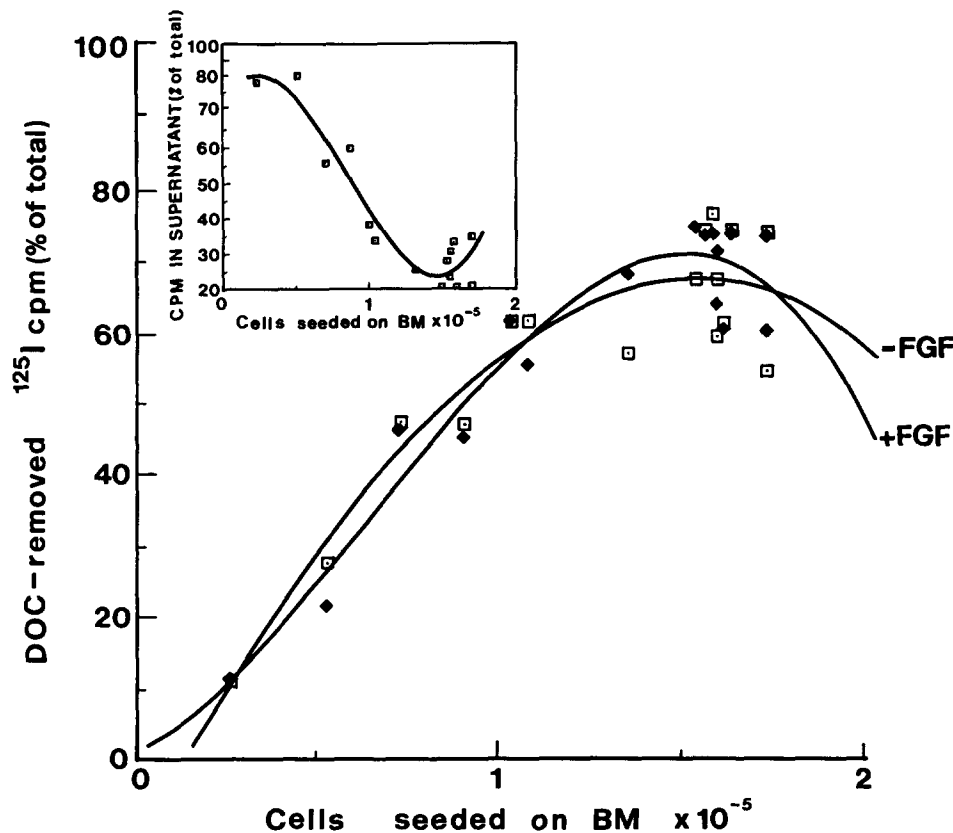
BCE cells were isolated from the adrenal cortex of yearling calves and grown as previously described (Folkman et al., 1979). The cells were routinely subcultured at a 1:5 split ratio and grown to confluency in 100-mm gelatin-coated plastic petri dishes in DME supplemented with 5% donor calf serum (growth medium). The BCE cells used in all the amnion assays described were derived from one clone and were used between passages 7 and 14.

### Cell Labeling with <sup>125</sup>I-Iododeoxyuridine

Subconfluent cultures of BCE cells, 2 d after trypsinization, were incubated at 37°C for 18 h in the presence of 3.0 μCi/ml of <sup>125</sup>I-iododeoxyuridine (2,200 Ci/mmol; New England Nuclear, Boston, MA) in growth medium. At the end of the incubation the cultures were washed three times with 10 ml of medium, trypsinized, and resuspended in 40 ml of growth medium. The cells were sedimented at 500 g for 5 min and resuspended in fresh growth medium containing penicillin (1,000 U/ml), streptomycin sulfate (40 mg/ml), and amphotericin B (Fungizone) (2.5 μg/ml). 10-μl aliquots of the cell suspension, labeling medium, pooled washings, trypsin, and supernatant after centrifugation were collected for determination of the radioactivity in a gamma counter (Packard Instrument Co., Inc., Downers Grove, IL). Cell concentration was determined using a hemocytometer. Under these conditions the radioactivity associated with the cells was 20–45% of the total radioactivity in the labeling medium. The nonsedimentable radioactivity in the cell suspension was 0–1%, and the specific labeling, determined in 27 experiments, was 1.98 ± 0.16 cpm/cell.

### Assay for BCE Cell Invasion into the Human Amniotic Membrane

Human amniotic membranes were isolated from fresh term placentas, fastened to Teflon rings, and denuded of the epithelium as previously described (Mignatti et al., 1986). The membranes were stored at 4°C in DME supplemented with antibiotics and Fungizone (as above) and were always used within 1 wk after preparation. In each assay tissue obtained from only one placenta was used. The Teflon rings holding the amnion membrane were placed onto silicone rubber rings fastened to the bottom of 35-mm microtiter plastic tissue culture wells (Costar, Data Packaging Corp., Cambridge, MA) with sterilized nontoxic silicone lubricant (Dow Corning Corp., Midland, MI). The inner diameter of the silicone rubber rings (lower compartment) was filled with 500 μl of growth medium containing antibiotics and Fungizone. The <sup>125</sup>I-iododeoxyuridine-labeled BCE cell suspension was added in the upper compartment of the amnion chamber in a final volume of 1 ml. In a typical assay, 1.5 × 10<sup>5</sup> BCE cells, corresponding to ~3.0 × 10<sup>5</sup> cpm <sup>125</sup>I were added in each invasion chamber. 3 ml of growth medium containing antibiotics and Fungizone were pipetted into the tissue culture well (outer compartment) so that the level of fluid was the same inside and outside the Teflon ring and no hydrostatic pressure would be exerted on the amniotic membrane. When indicated, bFGF was added to the cell suspension in the upper compartment, diluted in 10 μl of growth medium. When



**Figure 1.** Correlation between number of BCE cells seeded on the amnion BM and number of BM-attached cells. The graph reports data combined from 15 experiments in which  $^{125}\text{I}$ -iodo-deoxyuridine-labeled BCE cells were seeded on the amnion BM at the concentrations indicated, in the presence or in the absence of bFGF. In most experiments 70 ng/ml of bFGF were added in the lower chamber compartment as described in Materials and Methods. After 72 h incubation at  $37^\circ\text{C}$ , the culture medium was removed and the distribution of radiolabeled cells determined as described in Materials and Methods. The radioactivity associated with the pooled DOC and PBS washings is reported as percent of the total radioactivity recovered in the invasion chamber. In the inset, the radioactivity present in the culture supernatant (medium in the upper compartment) of samples run in the absence of bFGF is also reported as percent of the total  $^{125}\text{I}$  cpm recovered in the invasion chamber. As discussed in Results, most of this ra-

dioactivity was accounted for by intact cells and was not different in the presence or in the absence of bFGF. In both graphs each point represents the mean of triplicate samples. The curves were determined by a computerized statistical program. They all fit to a polynomial correlation with a highly significant correlation coefficient ( $r = 0.96$ ;  $p < 0.001$ ).

the growth factor was added in the lower compartment, it was diluted in growth medium to a concentration sevenfold higher than the concentrations added in the upper compartment, and 500  $\mu\text{l}$  were added inside the silicone rubber ring before seeding the cell suspension in the upper compartment. This was done to compensate for dilution when the 3 ml of growth medium were added in the outer compartment. The proteinase inhibitors and antibodies to be tested were diluted to a concentration fivefold higher than the final concentration indicated, and 200  $\mu\text{l}$  were incubated on the amnion BM for 1 h at room temperature. The cell suspension was then added in 800  $\mu\text{l}$  of growth medium containing antibiotics and Fungizone. The tissue culture plates were placed in a moist chamber and incubated at  $37^\circ\text{C}$  in a 5%  $\text{CO}_2$  atmosphere. After 72 h incubation the supernatant was removed and, after two washings with PBS, the cell layer was lysed in 0.5 ml of 4% Na-deoxycholate (DOC) and removed by carefully scraping with a rubber policeman. The tissue was washed three times with PBS, and the DOC and PBS washings were pooled in the same tube. The amnion was eventually detached from the Teflon ring, and the radioactivity associated with it, as well as that of the medium in the upper, lower, and outer compartments, the PBS, and the pooled DOC and PBS washings, was measured in a gamma counter. To determine the number of invasive cells, the  $^{125}\text{I}$  cpm associated with the amnion in the presence of a  $0.4 \mu\text{m}$  pore filter, which account for background, cell-free radioactivity, were subtracted from the radioactivity associated with the amnion in the absence of the filter, and the difference was divided by the specific cell labeling ( $^{125}\text{I}$  cpm/cell), as determined at the beginning of the assay. The amount of radioactivity associated with the membrane in the presence of the filter never exceeded 0.20% of the total cpm. Samples and controls were assayed in triplicate.

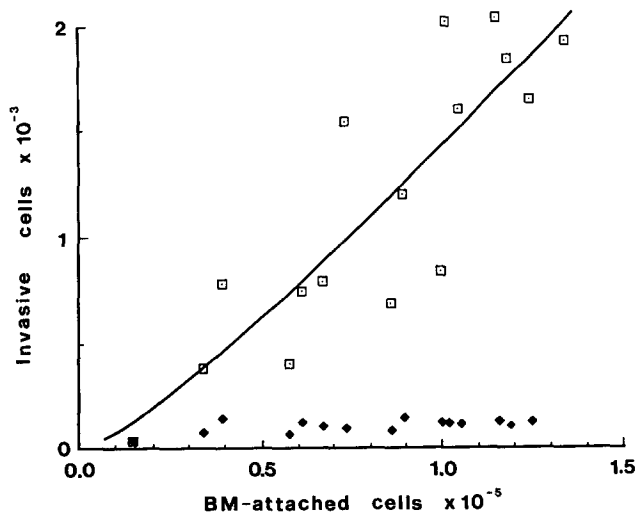
#### **bFGF Diffusion in the Amnion Invasion Chamber**

Two nanograms of  $^{125}\text{I}$ -labeled bFGF (4,500 cpm/ng; 70% TCA precipitable) were added either in the upper compartment or in the medium (500  $\mu\text{l}$ ) inside the silicone rubber ring in the lower compartment of amnion invasion chambers. Immediately after,  $1.50 \times 10^5$  BCE cells were seeded in

the upper compartment, and 3 ml of growth medium were added in the tissue culture well. At the end of incubation at  $37^\circ\text{C}$ , the medium in the tissue culture well was collected separately from the medium inside the silicone rubber ring, and the samples were processed as described above for amnion invasion assays. The media from the upper and lower compartments were TCA precipitated, and the radioactivity associated with them, as well as that of the PBS and DOC washings and of the amnion, was measured in a gamma counter. Triplicate samples were assayed.

#### **bFGF, Transforming Growth Factor Beta (TGF $\beta$ ), Proteinase Inhibitors, and Antibodies**

The human bFGF was purified from human placenta as previously described (Moscatelli et al., 1986). Recombinant bFGF, kindly provided by Synergen, Inc. (Boulder, CO), was also used in several experiments; its activity in the amnion invasion assay was equivalent to that of the placental bFGF. Anti-human bFGF IgG was precipitated with ammonium sulfate from rabbit antiserum raised against pure antigen by Dr. J. Joseph-Silverstein (New York University Medical School). Transforming Growth Factor beta was a gift from Dr. O. Saksela (University of Helsinki, Finland). Rabbit anti-human skin fibroblast collagenase antiserum was a gift from Dr. H. G. Welgus (Jewish Hospital, St. Louis, MO); purified anti-stromelysin and anti-type IV collagenase IgGs, previously shown to be monospecific (Wilhelm et al., 1987; Collier et al., 1988), were a gift from Dr. G. I. Goldberg (Washington University Medical Center, St. Louis, MO); rabbit nonimmune and anti-human tissue type plasminogen activator IgGs were purified from serum by ammonium sulfate precipitation and DEAE chromatography. Recombinant tissue inhibitor of metalloproteinases was a gift from Synergen, Inc. (Boulder, CO); Eglin was a gift from Dr. H. P. Schnabli (Ciba-Geigy, Basel, Switzerland); Erythrina trypsin inhibitor was a gift from Dr. E. L. Wilson (University of Cape Town, South Africa). 1-10 phenanthroline, soybean trypsin inhibitor, epsilon aminocaproic acid, and leupeptin were purchased from Sigma Chemical Co. (St. Louis, MO). Trasylol<sup>®</sup> was a gift from Bayer Werk (Elberfeld, West Germany).



**Figure 2.** Correlation between the number of BM-attached BCE cells and the number of cells that invade through the BM. The graph reports the data combined from 16 independent experiments in which  $^{125}\text{I}$ -iododeoxyuridine-labeled BCE cells were added on the amnion BM at different concentrations,  $0.25\text{--}1.70 \times 10^5$  cells per invasion chamber, and in the absence or in the presence of 70 ng/ml bFGF added in the lower compartment. After 72 h incubation at  $37^\circ\text{C}$ , samples and controls were treated as described in Materials and Methods. For determination of the number of BM-attached cells, the radioactivity associated with the pooled DOC and PBS washings was divided by the specific cell labeling, after subtraction of the  $^{125}\text{I}$  cpm associated with the DOC plus PBS washings of control samples in which a  $0.4\text{-}\mu\text{m}$  filter prevented cell attachment on the amnion BM. The number of invasive cells was determined as described in Materials and Methods. Each point represents the mean of triplicate samples. The curve was determined by a computerized statistical program. It fits to a polynomial correlation with a highly significant correlation coefficient ( $r = 0.86$ ;  $p < 0.001$ ). No significant correlation could be evidenced for the data obtained in the absence of bFGF.  $\square$ , 70 ng/ml bFGF;  $\blacklozenge$ , control.

## Results

### Characterization of BCE Cell Invasion into the Human Amniotic Membrane

Under the culture conditions described in Materials and Methods, the BCE cells grown on gelatin-coated dishes reached a density of  $7.5 \times 10^4$  cells/cm $^2$ . When confluent cultures were split 1:5, virtually 100% of the cells spread on the substrate and were usually confluent in 5–6 d. When  $1.5 \times 10^4$  BCE cells/cm $^2$  (corresponding to a confluent culture split 1:5) were seeded on the amnion BM, they spread rapidly and acquired a spindle-shaped morphology, but within 24 h after seeding most of the cells were found floating in the medium. However, when higher numbers of cells were added onto the amnion, their attachment was more stable. This observation was confirmed when  $^{125}\text{I}$ -iododeoxyuridine-labeled BCE cells were used. After 72 h incubation, the cells attached on the amnion BM were removed by lysis with 4% DOC and scraping with a rubber policeman, as described in Materials and Methods. The percent of the total applied radioactivity associated with the DOC and subsequent PBS washings provided a measurement of the number of cells that were attached on the BM. Fig. 1 shows a compilation of

results obtained in different experiments in which the cells were seeded at different densities. The percent of DOC-removed radioactivity correlated with the number of cells added onto the amnion BM. The correlation was highly significant ( $p < 0.001$ ) and showed that the percent of BM-attached cells increased for cell densities ranging from  $2.25 \times 10^4$  to  $1.50 \times 10^5$  cells per invasion chamber (2 cm $^2$ ). For higher cell densities, corresponding to overconfluence, the slope of the regression line leveled off and then tended to become negative. This indicated that under these conditions the number of cells that remained stably attached to the amnion BM increased with increasing the cell density, and decreased when the cells were added at a density corresponding to overconfluence. The maximum radioactivity associated with the DOC washing was 70–75% of the total  $^{125}\text{I}$  input. As the radioactivity removed by DOC increased, the percent of radioactivity in the medium of the upper compartment decreased (Fig. 1, inset). 60–70% of this radioactivity was sedimentable, indicating that it primarily represented intact cells. Addition of bFGF in the upper or in the lower compartment of the invasion chamber did not significantly affect cell attachment. As shown in Fig. 1, the correlation lines between the number of cells seeded and the percent of radioactivity removed by DOC in the presence and in the absence of bFGF were superimposable.

We then verified whether the BCE cells were able to invade into the amnion stroma by measuring the radioactivity associated with the membrane after the attached cells had been removed. To measure possible contamination of the membrane by cell-free radioactivity liberated during cell lysis and subsequent scraping of the amnion BM,  $^{125}\text{I}$ -iododeoxyuridine-labeled BCE cells were grown in control chambers on a  $0.4\text{-}\mu\text{m}$  pore polyester filter placed on the membrane surface. As was previously described, the filter prevented the cells from invading but was permeable to soluble cellular components (Mignatti et al., 1986). By this method, the number of cells remaining in the amnion stroma after the quantitative removal of the cells growing on the BM could be determined as described in Materials and Methods. The value obtained for the number of cells present in the amnion stroma gave a lowest estimate, and would differ from the actual number of invasive cells depending on the decrease in specific activity caused by cell growth. As shown in Fig. 2, significant amnion invasion by the BCE cells could be detected by this method. Invasion depended on the presence of bFGF, and the number of invasive cells was a function of the number of cells attached to the amnion BM. In the presence of 70 ng/ml bFGF in the lower compartment, the estimated number of invasive cells after 72 h incubation increased from 300 per chamber to  $>2,000$  per chamber, when the cell density increased from  $3.20 \times 10^4$  to  $1.50 \times 10^5$  cells per chamber. This is approximately the same proportion of cells found to be invasive when B16/BL6, an invasive mouse melanoma cell line, was previously examined (Mignatti et al., 1986). On the contrary, in the absence of bFGF the estimated number of BCE cells in the amnion stroma never exceeded 70–120 at all cell densities. The correlation between the number of BM-attached and invasive cells in the presence of bFGF was highly significant ( $p < 0.001$ ). In the absence of the growth factor no significant correlation was evident. These results indicated that (a) BCE cell attachment to the amnion BM depended on the density at which the cells were added in the

**Table I, A and B. Reproducibility of the Amnion Invasion Assay with BCE Cells**

| <b>A</b>                        |                                      |                              |                                      |                              |                                      |                              |
|---------------------------------|--------------------------------------|------------------------------|--------------------------------------|------------------------------|--------------------------------------|------------------------------|
| Experimental conditions         | Experiment 1                         |                              | Experiment 2                         |                              | Experiment 3                         |                              |
| N. seeded cells                 | 150,000                              |                              | 150,000                              |                              | 155,000                              |                              |
| <sup>125</sup> I cpm/cell       | 2.77                                 |                              | 1.56                                 |                              | 1.29                                 |                              |
| Total <sup>125</sup> I cpm*     | 378,521 ± 28,389                     |                              | 223,846 ± 15,445                     |                              | 185,954 ± 13,575                     |                              |
| <b>B</b>                        |                                      |                              |                                      |                              |                                      |                              |
| Additions                       | <sup>125</sup> I cpm‡<br>(mean ± SD) | N. invasive<br>cells§ (mean) | <sup>125</sup> I cpm‡<br>(mean ± SD) | N. invasive<br>cells§ (mean) | <sup>125</sup> I cpm‡<br>(mean ± SD) | N. invasive<br>cells§ (mean) |
| 0.4 μm filter                   | 567 ± 251                            | 0.0                          | 358 ± 67                             | 0.0                          | 503 ± 96                             | 0.0                          |
| None                            | 785 ± 283                            | 78.7                         | 493 ± 22                             | 86.5                         | 589 ± 48                             | 86.7                         |
| bFGF 10 ng/ml<br>in upper comp. | 2,638 ± 440                          | 747.7                        | 1,366 ± 45                           | 646.2                        | 1,571 ± 227                          | 827.9                        |
| bFGF 70 ng/ml<br>in lower comp. | 5,698 ± 525                          | 1,852.5                      | 3,483 ± 347                          | 2,003.7                      | 2,762 ± 157                          | 1,751.2                      |

BCE cells were metabolically labeled with <sup>125</sup>I-iododeoxyuridine, as described in Materials and Methods, and the specific cell labeling was determined by calculating the ratio between <sup>125</sup>I cpm/ml of washed cell suspension and number of cells per milliliter as determined in a hemocytometer. The cells were then seeded on the amnion BM at the concentrations indicated. Details for addition of bFGF and computation of the number of invasive cells are described in Materials and Methods. Samples and controls were assayed in triplicate. The results obtained in three independent experiments are reported.

\* Total <sup>125</sup>I cpm recovered per invasion chamber, calculated by summing the radioactivity present in upper and lower chamber medium, PBS and DOC plus PBS washings, amnion membrane, and polyester filter (when present). Mean ± SD is reported.

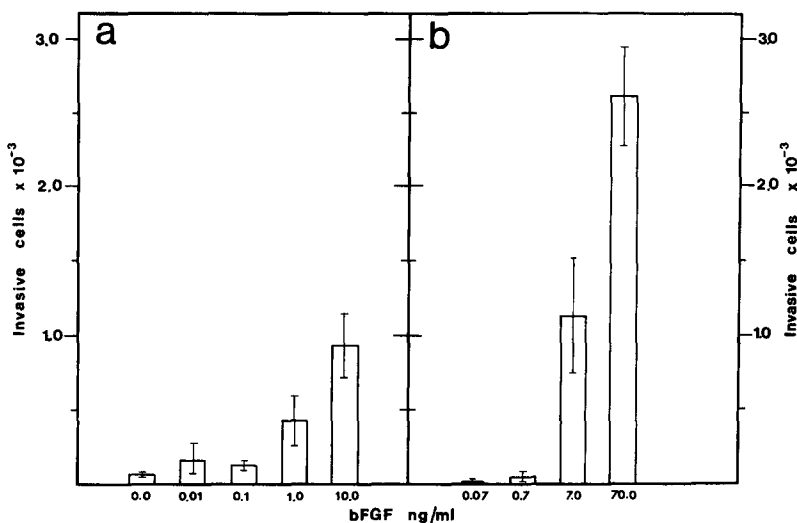
‡ Amnion-associated radioactivity after DOC treatment, scraping, and PBS washings.

§ Estimated number of invasive cells, calculated as reported in Materials and Methods.

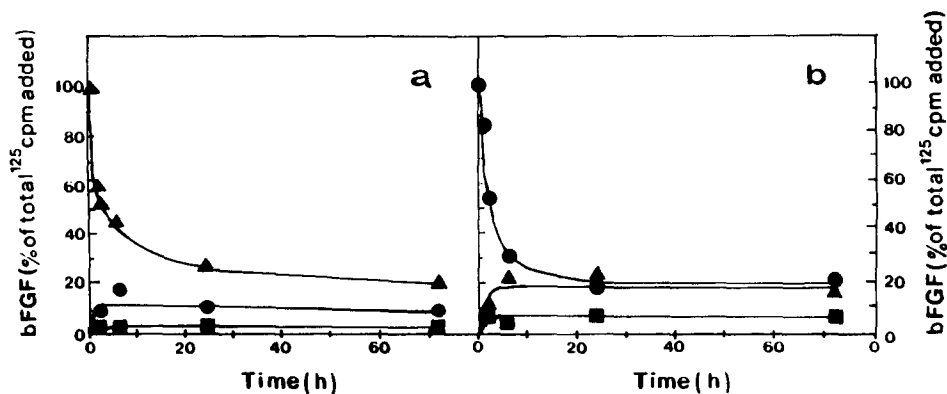
invasion chamber and was not affected by bFGF, and (b) BCE cell invasion through the amnion BM depended both on the cell number and on the presence of bFGF, and was maximal when the cells on the BM were confluent. The latter condition was attained when  $1.50 \times 10^5$  BCE cells were added onto the amnion BM. Under these experimental conditions the results obtained by the assay appeared very reproducible. As shown in Table I, the calculated number of cells present in the amnion stroma after 72 h incubation was comparable in different experiments, regardless of the specific cell labeling at the beginning of the assay and of the total <sup>125</sup>I cpm input in the invasion chamber.

The effect of bFGF on BCE cell invasion into the amniotic membrane was dose dependent, regardless of whether the growth factor was added in the upper or in the lower compartment of the invasion chamber. As shown in Fig. 3,

$1.0-2.5 \times 10^3$  cells per chamber, invaded into the amnion in the presence of 10-70 ng/ml bFGF. As described in Materials and Methods, excess bFGF was added in the lower compartment to compensate for dilution in the total volume of medium. Experiments done with <sup>125</sup>I-labeled bFGF showed that the growth factor diffused very rapidly through the amniotic membrane. As shown in Fig. 4, equilibrium between the upper and lower compartments was reached after 24 h incubation, either when bFGF was added in the upper compartment (Fig. 4 a) or when bFGF was added in the lower compartment (Fig. 4 b). In the latter case, the concentration gradient obtained between the two compartments during the initial 24 h of the assay was steeper than when bFGF was added in the upper compartment. This was probably because the initial bFGF concentration in the lower compartment was twice as high as in the upper compartment (4 ng/ml vs.



**Figure 3.** Dose-dependent stimulation of BCE cell amnion invasion by bFGF added in the upper or in the lower compartment of the invasion chamber. (a) bFGF added in the upper compartment: the growth factor was added immediately after seeding the <sup>125</sup>I-iododeoxyuridine-labeled BCE cell suspension. (b) bFGF added in the lower compartment: the growth factor was added in the lower compartment of the invasion chamber as described in Materials and Methods. The two experimental conditions were tested in parallel assays in which  $1.5 \times 10^5$  <sup>125</sup>I-iododeoxyuridine-labeled BCE cells were added on the amnion BM. After 72 h incubation at 37°C the samples were processed and the number of invasive cells per invasion chamber was determined as described in Materials and Methods. Mean and standard error of three experiments are shown.



**Figure 4.** Diffusion of  $^{125}\text{I}$ -labeled bFGF through the amniotic membrane and BCE cell layer. Two nanograms of  $^{125}\text{I}$ -labeled bFGF (4,500 cpm/ng) were added either in the upper compartment (a) or in the medium (500  $\mu\text{l}$ ) inside the silicone rubber ring in the lower compartment (b) of amnion invasion chambers. Immediately after,  $1.50 \times 10^5$  BCE cells were seeded in the upper compartment, and 3 ml of growth medium were added in the tissue culture well. The chambers were incubated at

37°C and at the time points indicated, the medium in the different compartments was collected, TCA precipitated, and the radioactivity measured in a gamma counter. Each point represents the mean of triplicate samples.  $\blacktriangle$ , upper compartment;  $\bullet$ , lower compartment;  $\blacksquare$ , outer compartment.

2 ng/ml, respectively). The bFGF associated with the BCE cell layer and the amnion under either condition of addition accounted for 5–10% of the total bFGF input. On the contrary, the bFGF diffusing into the outer compartment represented no more than 15% of the amount of bFGF remaining inside the lower compartment and accounted for  $\sim 3\%$  of the total bFGF input.

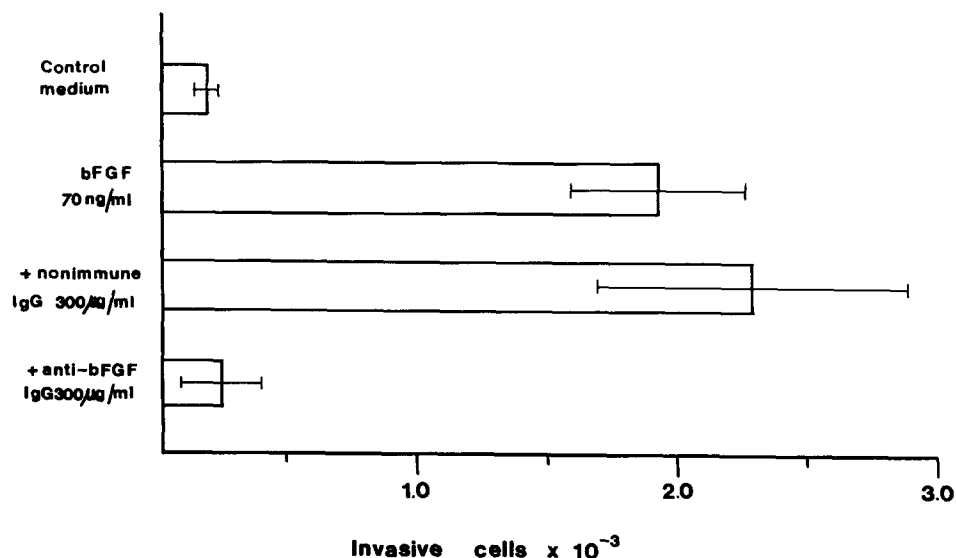
As shown in Fig. 5 the effect of bFGF was neutralized by a specific rabbit anti-human bFGF IgG but not by nonimmune rabbit IgG. Moreover, the stimulation of BCE cell invasion was reproduced using recombinant bFGF made in *Escherichia coli*, thereby demonstrating that the effect resulted from the action of bFGF and not from a contaminant.

Phase contrast microscopy provided further evidence of the effect of bFGF on the BCE cells grown on the amnion BM. BCE cells grown at high density on this substrate appeared to form a regular monolayer of closely apposed elongated cells (Fig. 6 a). After 72 h incubation in the presence of 70 ng/ml bFGF in the lower compartment, some cells showed a more irregular morphology, with branching processes criss-crossing the regularly oriented monolayer (Fig.

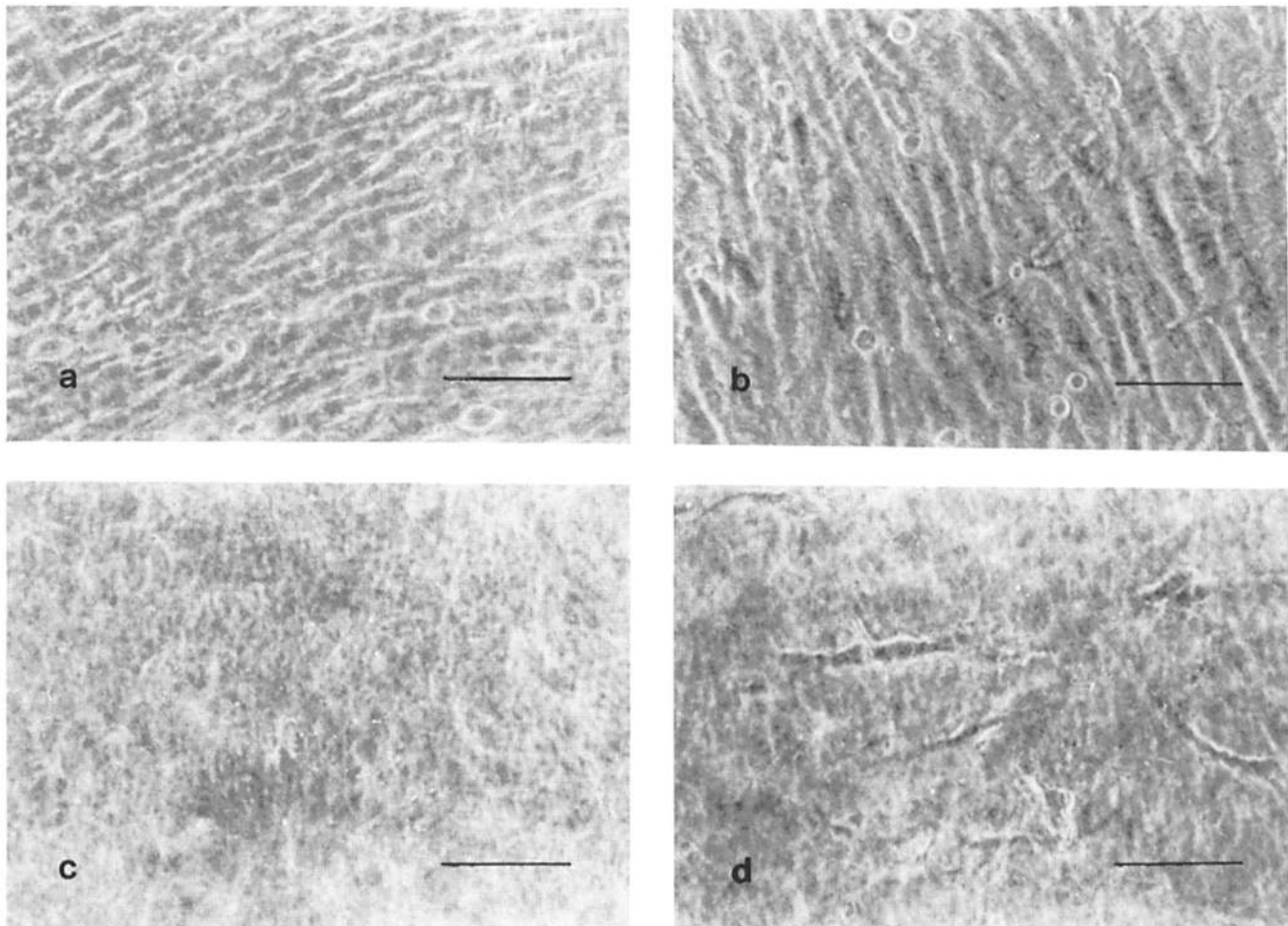
6 b). While no cells were visible in the stroma in the absence of bFGF (Fig. 6 c), refractile elongated cells could be seen dispersed in the irregular array of the stromal collagen fibers below the BM (Fig. 6 d). Due to the extreme thickness of the endothelial cell layer and underlying amnion, however, the precise nature of these structures was not clear. Occasionally, when cut edges of the amnion were examined by scanning electron microscopy, very long tube-like structures could be seen in the stroma. These structures, which were several hundred micrometers in length and appeared to be multicellular, may represent capillary-like sprouts invading through the stroma. However, they were not found reproducibly, and their formation may require specific conditions.

#### **Effect of the TGF $\beta$ on BCE Cell Invasion through the Amnion BM**

TGF  $\beta$  has been reported to inhibit collagenase and urokinase plasminogen activator (uPA) activities in different cell types, including BCE cells, by decreasing the synthesis of these enzymes and simultaneously stimulating TIMP and



**Figure 5.** Inhibition of bFGF-induced BCE cell amnion invasion by anti-bFGF antibody. 70 ng/ml of bFGF were added in the lower compartment as described in Materials and Methods. Purified anti-human bFGF or nonimmune rabbit IgG were preincubated on the BM as described in Materials and Methods. The concentrations indicated are the concentrations in the final volume of medium (1 ml) after addition of the cell suspension. After 72 h incubation at 37°C samples and controls were processed and the number of invasive cells per invasion chamber was determined as described in Materials and Methods. Mean and standard error of three experiments are shown.



**Figure 6.** Morphology of BCE cells grown on the amnion BM. 150,000 BCE cells were seeded on the amnion BM in the absence or in the presence of 70 ng/ml bFGF added in the lower chamber compartment as described in Materials and Methods. After 72 h incubation at 37°C the cultures were photographed with an inverted phase contrast microscope focusing at different depths. (a) Cells in the absence of bFGF; focus on the cell layer. (b) Cells in the presence of bFGF; focus on the cell layer. (c) Cells in the absence of bFGF; focus in the stroma. (d) Cells in the presence of bFGF; focus in the stroma. Bars, 10  $\mu$ m.

type 1 PA inhibitor (PAI-1) production (Sporn et al., 1986; Lahio et al., 1986a, Edwards et al., 1987; Lund et al., 1987; Saksela et al., 1987). Moreover, it has also been shown to inhibit the proliferation and motility of BCE cells and to prevent their invasion into collagen matrices (Muller et al., 1987). It was, therefore, of interest to test whether TGF  $\beta$  inhibited BCE cell invasion into a more complex structure, such as the amniotic membrane. TGF  $\beta$  was added in the medium of the upper compartment after the cells had been seeded on the BM and stimulated to invade through the addition of 70 ng/ml bFGF in the lower compartment. As shown in Fig. 7, TGF  $\beta$  had a dose-dependent inhibitory effect on BCE cell invasion. 1 and 10 ng/ml prevented tissue invasion, whereas 0.1 ng/ml was ineffective. At the concentrations tested, TGF  $\beta$  did not appear to be cytotoxic nor to interfere with cell attachment to the amnion BM, as judged from the amount of radioactivity recovered in the medium of the upper compartment compared to that of the control chambers.

#### **Effect of Proteinase Inhibitors on BCE Cell Invasion through the Amnion BM**

It has been proposed that during angiogenesis, as well as in

tumor invasion, the proteinases produced by the cells play a central role in the degradation of and infiltration into the BM by invasive cells (Gross et al., 1983; Mullins and Rohrlach, 1983; Danø et al., 1985; Mignatti et al., 1986; Moscatelli and Rifkin, 1988). To test this hypothesis we used the assay described above and attempted to inhibit selectively specific enzymes with either pure proteinase inhibitors or antibodies. Since bFGF is known to stimulate collagenase and PA production by BCE cells (Moscatelli et al., 1986; Presta et al., 1986), attention was focused on these proteinases. 150,000 <sup>125</sup>I-iododeoxyuridine-labeled BCE cells were added on the amnion BM and stimulated to invade by the addition of 70 ng/ml bFGF on the stromal aspect of the tissue. To facilitate enzyme-inhibitor interactions at the cell substratum contact sites, the proteinase inhibitors and antibodies were preadsorbed on the amnion BM for 1 h at room temperature, as described in Materials and Methods.

The role of serine proteinases in BCE cell invasion was studied by using inhibitors of plasmin. These included aprotinin (trasyolol), soybean trypsin inhibitor, epsilon aminocaproic acid (EACA), Erythrina trypsin inhibitor, a natural inhibitor of the tissue type plasminogen activator (tPA) that also inhibits plasmin (Heussen et al., 1984), and leupeptin,



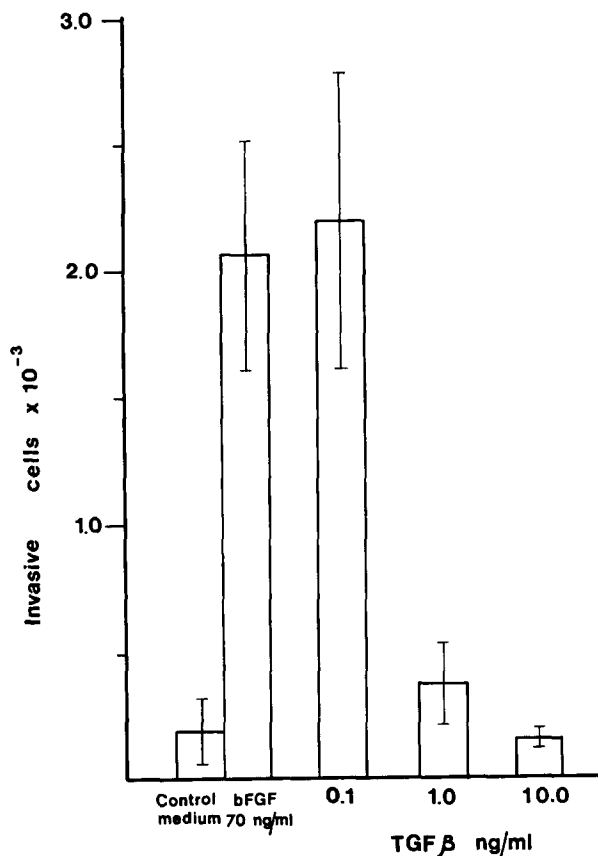


Figure 7. Dose-dependent inhibition of bFGF-induced BCE cell amnion invasion by TGF  $\beta$ . 70 ng/ml of bFGF were added in the lower chamber compartment as described in Materials and Methods, before seeding  $1.50 \times 10^5$   $^{125}$ I-iododeoxyuridine-labeled BCE cells in the upper compartment. In addition, control medium or the indicated concentrations of TGF  $\beta$  were added to the cell suspension in 10  $\mu$ l of growth medium immediately after seeding. After 72 h incubation at 37°C samples and controls were processed as described in Materials and Methods. The number of invasive cells per invasion chamber was estimated as described in Materials and Methods. Mean and standard error of three experiments are shown.

an inhibitor of serine and cysteine proteinases. Anti-human tPA-purified IgG, which inhibited bovine tPA, was also tested. Moreover, Eglin was used in these experiments as an inhibitor of elastase. As shown in Fig. 8, the plasmin inhibitors tested inhibited amnion invasion by the BCE cells. Interestingly, the soybean trypsin inhibitor was not as effective as trasylol, EACA, and the Erythrina inhibitor, and did not completely inhibit BCE cell invasion at a concentration that inhibited 100% of the plasmin generated by BCE cells in vitro (data not shown). Leupeptin, on the contrary, was totally ineffective. The failure of leupeptin to block cell invasion has been described earlier using mouse melanoma cells (Mignatti et al., 1986; Persky et al., 1986) and probably results from the low affinity of this inhibitor for plasmin. The anti-tPA IgG also prevented BCE cell invasion. On the contrary, Eglin as well as nonimmune serum and IgG were ineffective. None of these inhibitors and antibodies increased the amount of radioactivity in the cell supernatant above control values. Therefore, their inhibitory activity was not due

to a cytotoxic effect nor to interference with BCE cell attachment to the amnion BM.

We then studied the role of metalloproteinases in BCE cell invasion by testing the effect of metalloproteinase inhibitors and/or anti-collagenase antibodies. The reagents tested included 1-10 phenanthroline, recombinant tissue inhibitor of metalloproteinases, an anti-human skin fibroblast interstitial collagenase antiserum, anti-basement membrane (type IV) collagenase (Collier et al., 1988), and anti-stromelysin purified IgGs. At the concentrations indicated, none of these reagents appeared to be cytotoxic or appeared to prevent BCE cell attachment to the BM. Only in the presence of 1-10 phenanthroline was the amount of radioactivity in the cell supernatant higher than in control chambers ( $\sim 40$  vs. 20% of the total cpm, respectively). Most of this radioactivity was sedimentable, indicating that this reagent interfered with BCE cell attachment to the amnion BM. As shown in Fig. 9 each of the metalloproteinase inhibitors and antibodies tested, except the anti-stromelysin IgG, was able to totally block BCE cell invasion into the amnion. Nonimmune serum and IgG were ineffective.

## Discussion

The formation of new capillaries consists of several components, including cell multiplication at the base of the sprouting vessel, local degradation of the capillary basal lamina, migration of endothelial cells into the surrounding stroma, and the eventual differentiation of the migrated cells into mature microvessels (Ausprunk et al., 1975; Ausprunk and Folkman, 1977; Folkman, 1986). bFGF has been shown to stimulate all of these components of angiogenesis (Moscattelli et al., 1986; Presta et al., 1986). In this process the degradation of the basal lamina is of central importance, since it permits endothelial cell infiltration into the surrounding stroma and migration towards the source of the angiogenic inducer. The basal lamina does not contain preformed channels that could afford cell migration, and in most cases angiogenesis initiates in the absence of any discontinuity in the tissue. Thus capillary endothelial cells must degrade the BM to infiltrate into the surrounding tissue. The complex structure and composition of the microvascular extracellular matrix suggest that multiple and highly specialized cell functions are required for its degradation. Endothelial cells have been shown to produce enzymes capable of degrading extracellular matrix proteins (Kalebic et al., 1983; Laug et al., 1985; Pepper et al., 1987). The analysis of this process in vivo, under the experimental conditions of most angiogenesis assays, is difficult and attempts have been made to establish in vitro angiogenesis assays (Nicosia et al., 1983; Laug et al., 1985; Montesano and Orci, 1985, 1987; Montesano et al., 1983, 1986, 1987). Microvascular endothelial cells cultured on the human amnion BM provide an in vitro model for the capillary wall. Under these relatively defined conditions, BM degradation and endothelial cell infiltration into the interstitial stroma can be analyzed without the interference of host factors.

The experiments presented in this paper have revealed several features of BCE invasion of the amnion. First, cell invasion was dependent on cell density and appeared to be maximal under optimal conditions for cell attachment. While BCE cells can be grown at low densities on gelatin-coated



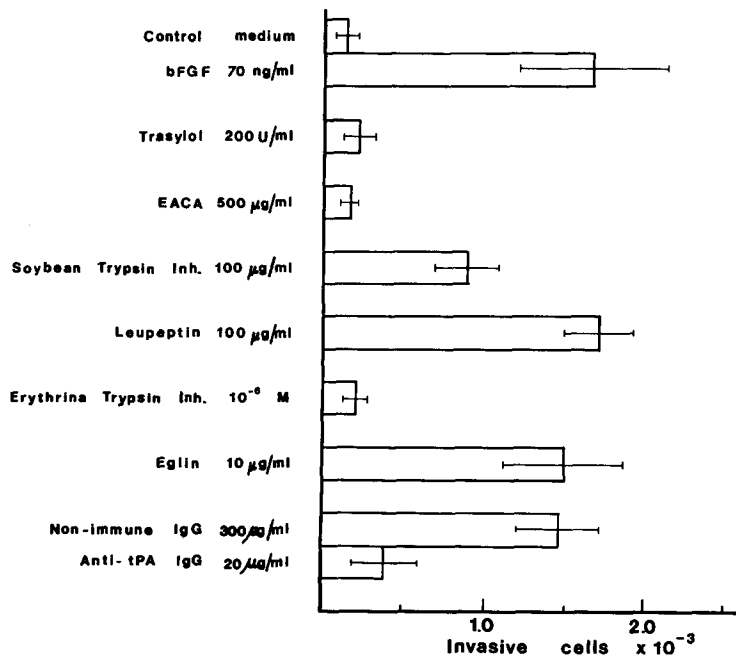


Figure 8. Effect of serine-proteinase inhibitors and anti-plasminogen activator antibodies on bFGF-induced BCE cell amnion invasion. 70 ng/ml of bFGF were added in the lower compartment as described in Materials and Methods. In addition, control medium or the inhibitors and IgGs indicated were preincubated on the amnion BM as described in Materials and Methods, before seeding  $1.50 \times 10^5$  <sup>125</sup>I-iododeoxyuridine-labeled BCE cells. After 72 h incubation at 37°C samples and controls were processed and the number of invasive cells per invasion chamber was determined as described in Materials and Methods. Mean and standard error of five experiments are shown.

plastic dishes, stable attachment and spreading on the amnion BM appeared to be directly proportional to the density at which the cells were seeded. Perhaps, the cell-to-cell contact achieved at high cell density is more representative of the conditions in the vessel wall and is more inductive for certain cellular responses than when cells are at low densities. Cell morphology appeared different on the amnion BM than on gelatin-coated dishes. These morphological changes may indicate an important role for BM components in modulating BCE cell behavior. Previous data have shown that the growth rate of these cells depends on the type of collagen on which they are grown, and that on basement membrane (type IV) collagen microvascular endothelial cells differentiate into tube-like structures (Madri et al., 1983; Montesano et al., 1983). Second, BCE cell invasion was dependent upon the addition of bFGF to the amnion assay. In the absence of bFGF essentially no cells were found in the amnion. Addition of bFGF to the cell supernatant or to the stromal aspect of the amnion induced invasion in a dose-responsive manner. The bFGF diffusion experiments showed that bFGF, with a molecular mass of 18 kD, readily permeates the human amniotic membrane, which has a molecular mass cut-off of 60 kD (Liotta et al., 1980). Third, invasion required the activities of both serine- and metalloproteinases.

The results obtained with serine proteinase inhibitors point to a central role for plasmin in BCE cell invasion. Inhibition of plasmin activity or plasmin formation totally blocked amnion invasion. However, not all of the plasmin inhibitors tested showed the same inhibitory effect on BCE cell invasion. Soybean trypsin inhibitor was not as effective as EACA, trasylol, or the Erythrina trypsin inhibitor, whereas leupeptin was totally ineffective. While soybean trypsin inhibitor, EACA, and trasylol have been shown to inhibit tumor cell invasion, the failure of leupeptin in blocking invasion has also been reported in other experimental systems (Mignatti et al., 1986; Persky et al., 1986). A possible explanation for the lack of inhibitory activity by these two inhibitors may be

that they inhibit plasmin less effectively than other specific inhibitors under the experimental conditions used. To effectively block invasion, inhibition of proteinase activity must occur locally at cell substratum contact sites. Recent data have shown the presence of cell surface receptors for PAs and plasminogen (Quigley, 1976; Hoal et al., 1983; Bajpai and Baker, 1985; Miles and Plow, 1985; Vassalli et al., 1985; Plow et al., 1986; Stoppelli et al., 1986; Pöllänen et al., 1988). The enzymes bound to their specific receptors appear to be at least partially protected from inactivation by proteinase inhibitors (Chapman et al., 1982; Chapman and Stone, 1984; Knudsen et al., 1986; Plow et al., 1986). Natural inhibitors have been shown to bind to extracellular matrix and to be active in this form (Laiho et al., 1986; Knudsen et al., 1987; Levin and Santell, 1987; Mimuro et al., 1987; Pöllänen et al., 1987). Therefore, complex interactions occur at the sites of proteolysis by invading cells. Invasion of collagen or fibrin gels by PMA-stimulated BCE cells has been shown not to require plasmin activity (Montesano and Orci, 1985; Montesano et al., 1987). The interpretation of these results is limited by the artificial conditions of the systems used. The amnion BM consists of a complex array of substrates, including several major glycoproteins, such as fibronectin and laminin, and heparan sulfate proteoglycans. Plasmin may play a central role in the degradation of these substrates, as well as in the activation of procollagenase(s).

The results obtained using anti-tPA IgG raise an interesting question as to the proteolytic requirements for plasminogen activation by BCE cells. Capillary endothelial cells have been reported to produce only tPA in vivo, and both tPA and uPA upon passaging in culture (Kristensen et al., 1984; Levin and Loskutoff, 1982; Moscatelli, 1986; Van Hinsbergh, 1987). The clone of BCE cells we used in these experiments was shown by zymography to produce both uPA and tPA. The former was prevalent in cell extracts, whereas cell-conditioned medium contained only tPA (data not shown). While we were able to demonstrate a requirement

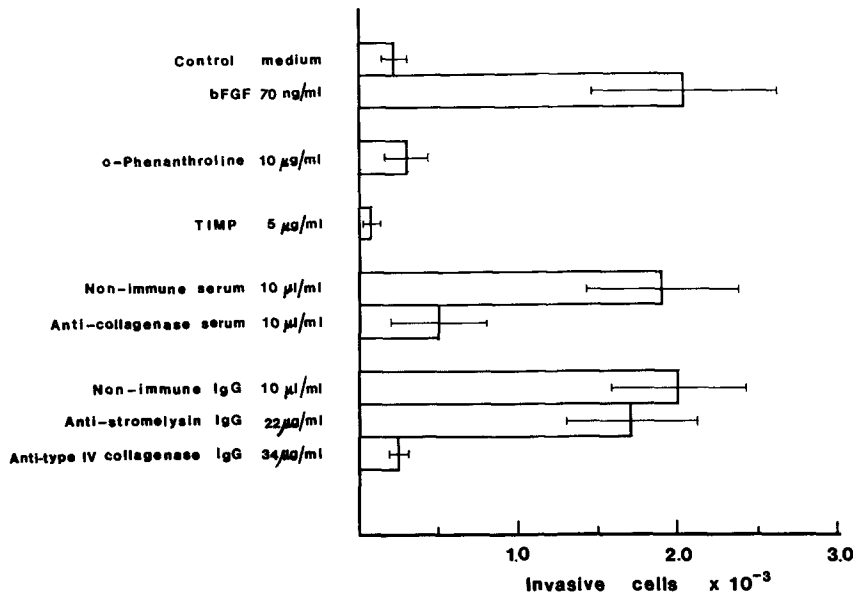


Figure 9. Effect of metalloproteinase inhibitors and anti-collagenase antibodies on bFGF-induced BCE cell amnion invasion. Experimental conditions were the same as described in the legend to Fig. 8. Mean and standard error of five experiments are shown.

for tPA by the use of anti-human tPA antibodies that cross react with bovine tPA, we were unable to do the identical experiment for uPA since the neutralizing potential of anti-human uPA antibodies for bovine uPA is weak. This limited our investigation of the potential role of the uPA produced by BCE cells in amnion invasion. A more complete description of this process will require the availability of selective antisera against bovine uPA, as well as a better understanding of the activities of PAs in solid substrate assays such as the amnion. Regardless of this limitation, our results stress the importance of the PA-plasmin system in BCE cell invasion of the BM.

The data obtained using metalloproteinase inhibitors also demonstrated a role for these enzymes in amnion invasion by BCE cells. Recombinant tissue inhibitor of metalloproteinases, as well as 1-10 phenanthroline, prevented the BCE cells from invading into the amnion. Moreover, the collagenolytic requirements for BCE cell invasion appear to be very specific. Anti-type IV and anti-interstitial collagenase antibodies completely prevented amnion invasion. On the contrary, anti-stromelysin antibody was ineffective, as was Eglin, an inhibitor of elastase. While stromelysin can degrade several BM and stromal components, including type IV collagen, fibronectin and laminin (Wilhelm et al., 1987), and certain elastases can degrade collagen(s) in its nonhelical structure (Mainardi et al., 1980; Pipoly and Crouch, 1987), these enzymes do not appear to be crucial for BM invasion by BCE cells. Stromelysin has recently been shown to activate procollagenase in vitro (Murphy et al., 1987). The failure of anti-stromelysin antibody to inhibit BM invasion indicates that in this experimental model other pathways of procollagenase activation may be effective.

The inhibition of invasion obtained with anti-type IV collagenase makes an interesting point as to the importance of this enzyme in invasive processes. Type IV collagen is the major protein component of BMs (Liotta et al., 1983; Madri et al., 1983; Yurchenco and Ruben, 1987), and its degradation appears to play a central role in cell invasion. However,

complete inhibition of BCE cell invasion was also obtained by anti-interstitial collagenase antibody. While this effect may be explained by a limitation of the assay, in that cells located superficially in the amnion may be removed by DOC lysis and scraping, it also points to the potential importance of interstitial collagenases in tissue invasion. Cells that have degraded the BM may in fact be unable to invade into the stroma if they lack specific degradative enzymes for interstitial collagens.

Thus, the invasive process that occurs during bFGF-stimulated BCE cell invasion of the human amnion BM requires the coordinated action of both the plasminogen activator-plasmin system and of specific collagenases. The question arises whether the inhibitory effect of proteinase inhibitors is due to inhibition of cell motility or to inhibition of matrix degradation. Recent unpublished data obtained with the migration assay described by Sato and Rifkin (1988) have shown little or no effect of several serine- and metalloproteinase inhibitors on capillary endothelial cell migration in vitro (Sato, Y., and D. B. Rifkin, personal communication). We can then conclude that the inhibition of capillary cell invasion by proteinase inhibitors is due to inhibition of extracellular matrix degradation.

The invasive process that occurs during angiogenesis has several features in common with tumor invasion. With both cell types it was found that ~1-2% of the cell population is able to invade through the amnion basement membrane in the invasion assay described (Mignatti et al., 1986). However, while tumor cells appear to be spontaneously invasive, capillary endothelial cells require the action of an angiogenesis factor, such as bFGF. Both tumor and capillary endothelial cells use a cascade of proteolytic enzymes for this process, which involves the coordinated action of both serine and metalloproteinases. We have previously shown that B16 mouse melanoma cells use uPA to initiate this proteolytic cascade, while capillary endothelial cells appear to use tPA. However, the final product, plasmin, is the same in both systems. Thus, the invasive mechanisms of tumor cells appear

not to be unique to the neoplastic state but rather represent the inappropriate expression of normal functions that may be shared by a variety of invasive cells. Since pericellular proteolysis is rigorously controlled via extracellular proteolytic inhibitors and enzyme-binding sites, it will be of interest in the future to establish how these additional control mechanisms may modify cell invasion.

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