Correlation of Cell Migration, Cell Invasion, Receptor Number, Proteinase Production, and Basic Fibroblast Growth Factor Levels in Endothelial Cells

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Abstract. The levels of endogenous basic fibroblast growth factor (bFGF) in seven clones of cultured bovine capillary endothelial (BCE) cells were assayed, and their relation to cell morphology, bFGF receptor number, cell migration, amniotic membrane invasivity, and proteinase levels were studied. Immunoblotting experiments with anti-bFGF IgG demonstrated that cells from these clones contained different amounts of bFGF. The cells containing high levels of bFGF had a spindle or elongated appearance at confluence and a low number of high affinity receptors for bFGF. The cells containing low levels of bFGF had a cobblestone-like appearance and a higher number of high affinity receptors. When exposed to 10 ng/ml bFGF, cells containing a low level of bFGF took on an elongated appearance with a crisscross pattern similar to that seen with the high producer bFGF cells.

The endogenous bFGF levels of the BCE cell clones correlated with the extent of cell migration after wounding of a monolayer and the degree of invasion of the human amniotic membrane. Cells from the clone with the highest endogenous bFGF level migrated well, invaded the amnion membrane without the addition of exogenous bFGF, and were relatively unaffected by the addition of bFGF. Cells from the clone containing the lowest level of bFGF did not migrate or invade under normal conditions. However, the addition of bFGF to the culture medium strongly enhanced both of these processes. The inclusion of anti-bFGF IgG in the media suppressed cell migration and invasion. The plasminogen activator (PA) activities of cell lysates of the clones, assayed by the 125I-fibrin plate technique, indicated that the PA levels did not correlate with the bFGF levels. Metalloproteinase activities in the conditioned medium, assayed by gelatin zymography, correlated with the endogenous bFGF levels, suggesting that the degree of expression of metalloproteinases might be critical for cell migration and invasion. These data suggest that endogenous bFGF may have an important role for migration and invasion of BCE cells during neovascularization via the induction and/or activation of specific metalloproteinases.

The formation of new capillaries, which takes place during embryonic development, wound healing, and malignant tumor growth, involves the migration and proliferation of capillary endothelial cells. Several polypeptides are known which induce angiogenesis (3). Among these proteins, basic fibroblast growth factor (bFGF) is one of the more potent angiogenic factors. The responses of capillary endothelial cells to bFGF in vitro include changes in cell morphology, stimulation of cell proliferation and migration, and increased production of proteinases such as plasminogen activator (PA) and collagenase (5, 7, 17, 23, 29, 31). We have recently shown that exogenous bFGF stimulates bovine capillary endothelial (BCE) cells to invade the human amniotic membrane and that this process is dependent upon the induction of proteinases since cell invasion is inhibited by specific inhibitors of serine and metalloproteinases (16).

Although bFGF had been thought to exert its effects on endothelial cells via a paracrine mode, it has recently been shown that endothelial cells produce high levels of bFGF (22, 34, 36, 37) and that endogenous bFGF is important for endothelial cell migration and PA production (32). During the characterization of several BCE cell clones from bovine adrenal cortices, we observed that the clones of BCE cells varied in their production of endogenous bFGF as well as their phenotypes. In this paper, we demonstrate that the endogenous bFGF levels correlate with cell morphology, receptor number, migration, invasion, and proteinase production. These data provide further evidence that bFGF can affect the cellular phenotype in an autocrine manner.

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1. Abbreviations used in this paper: aMEM, alpha minimum essential medium; BCE, bovine capillary endothelial; bFGF, basic fibroblast growth factor; PA, plasminogen activator; rbFGE, recombinant basic fibroblast growth factor.
Materials and Methods

Materials

bFGF was purified from term human placentas as described previously (23, 29). Recombinant bFGF (rbFGF) was a generous gift from Synergen, Inc. (Boulder, CO). Polyclonal antibodies against placental bFGF were raised in rabbits as described (10), and IgG fractions were prepared by either protein A-Sepharose column chromatography or bFGF affinity chromatography. This antibody recognized bFGF but no other heparin-binding proteins in Western blot or dot blot assays. rbFGF was diluted and stored in alpha minimum essential medium (αMEM) (Gibco Laboratories, Grand Island, NY) containing 0.1% gelatin. Human urokinase standard was purchased from Leo Pharmaceuticals (Copenhagen, Denmark).

Cell Culture

Seven clones of microvascular endothelial cells (8A2, 1, 8B4, 2-0, 8C1, 5A2, 5A5) were isolated from bovine adrenal cortices according to the method previously described (4, 6). For the first two passages after isolation, the cells were grown in conditioned medium from mouse sarcoma 180 cells. This was discontinued after the third passage. Each clone was derived from a single cell using cloning plates (4, 6). By convention, these cells are described as capillary endothelial cells although they may be pre- and post-capillary endothelial cells. The existence of factor VIII-related antigen in cells of the seven clones was confirmed by immunofluorescence (6). Cells were seeded on gelatin-coated dishes and grown in αMEM supplemented with 5% calf serum (Flow Laboratories, McLean, Virginia) with 2 mM penicillin. Culture medium was changed twice weekly. All experiments were seeded on gelatin-coated dishes and grown in αMEM supplemented with 0.1% gelatin (Gibco Laboratories) and 0.14 mg/ml streptomycin, 500 U/ml penicillin. Culture medium was changed twice weekly. All experiments were performed with cells between passages 9 and 14. Comparative studies with the clones were conducted with cells which differed by no more than one passage number. Unless otherwise noted, cells were always used at densities slightly below confluence.

Morphological Observations

Cells at confluence were washed twice with PBS, fixed with absolute methanol, and stained with Giemsa solution. Cells were observed and photographed with a phase-contrast microscope (E. Leitz, Inc., Wetzlar, FRG).

Protein Determination

Protein determinations were performed using the Pierce Chemical Co. (Rockford, IL) protein assay reagent according to the manufacturer’s instructions. A standard solution of BSA (Sigma Chemical Co. St. Louis, MO) including 0.5% SDS or 0.5% Triton X-100 in 0.1 M Tris-HCl buffer (pH 8.1) was used as a standard.

Plasminogen Activator Assay

BCE cells in 24-well dishes were incubated overnight in serum-free αMEM supplemented with 0.1% gelatin in the presence or absence of rbFGF, anti-bFGF IgG, or cell extracts. After washing twice with PBS, the cells were extracted with 0.5% Triton X-100 in 0.1 M Tris-HCl buffer (pH 8.1). Aliquots of cell extract (4.0 μg cell protein) were assayed on 127I-fibrin-coated plates as described previously (6). PA activity is presented in Ploug units using a urokinase standard.

Zymography for Metalloproteinases

Zymography for metalloproteinases was carried out using a modification of the method of Herron et al. (8, 9). BCE cells in 35-mm-diameter culture plates were incubated overnight in 1 ml serum-free αMEM. Gelatin dissolved in water (3 mg/ml) and 30% polyacrylamide were mixed and polymerized to make a final gel of 0.1% gelatin and 9% polyacrylamide. The amount of conditioned medium assayed was normalized to the total amount of cell protein determined after scraping and assay of the cells. Samples were loaded onto the gel without boiling or treatment with β-mercaptoethanol. After electrophoresis, the gel was cut into two pieces and soaked in 2.5% Triton X-100 solution for 1 h. One half of the gel was incubated overnight at 37°C in 2 mM CaCl2, 50 mM Tris-HCl buffer (pH 7.6), and the other half in the same buffer plus 10 mM EDTA. The gel was stained with 0.1% amido black. Prenatamed protein molecular weight standards (Bethesda Research Laboratories, Gaithersburg, MD) were used as markers.

Immunoblotting

After washing twice with PBS, subconfluent BCE cells were immediately scrapped in 0.5% SDS-containing PBS, boiled for 2 min, and sonicated. Cell lysates and a bFGF standard were loaded on an SDS–polyacrylamide gel with a 4% stacking gel and a 14% resolving gel (12). Upon completion of electrophoresis, the samples were transferred for 2 h onto a nitrocellulose membrane (Schleicher & Schuell Inc., Keene, NH) using an LKB Instruments Inc. (Bromma, Sweden) Multiphore II Nova blot system. The blots were probed with 200× diluted anti-bFGF IgG for 1 h at room temperature followed by incubation with 125I-protein A (New England Nuclear, Boston, MA). Competition experiments were carried out with the same amount of anti-bFGF IgG which was preincubated with 50 μg rbFGF for 1 h at room temperature. The molecular weight standards used in zymography were also used in this assay.

Receptor Assay

Labeling of rbFGF was performed with 125I (7 Ci/mg; New England Nuclear) using Iodo-Gen (Pierce Chemical Co.) as described previously (20, 25). The number of high affinity binding sites for 125I-bFGF on BCE cells was measured as described by Moscatelli (20). Cell number was computed by trypsinizing and counting cells from a pair of companion plates of each clone. Cells from each clone were plated in 60-mm dishes at a density that yielded slightly subconfluent cultures 4 d after plating. Cells were incubated with different concentrations of 125I-bFGF in serum-free αMEM supplemented with 0.1% gelatin for 2 h at 4°C. The 125I-bFGF bound to low affinity sites was removed with 2 M NaCl in Hepes (pH 7.5), while the 125I-bFGF bound to high affinity sites was removed with 2 M NaCl in acetate buffer (pH 4) (20). Receptor numbers were calculated by the method of Scatchard (33).

Cell Migration Assay

Cell migration was measured according to the method of Sato and Rifkin (33). Briefly, confluent monolayers of endothelial cells were wounded with a razor blade and incubated overnight at 37°C in 0.1% gelatin-containing αMEM with rbFGF or anti-bFGF IgG. After the incubation, the cells were stained and observed with a light microscope with an ocular grid. Migration was quantitated by counting the number of cells in successive 125-μm zones from the wound edge. Experiments were done twice, and the data shown are averages of eight random fields.

Invasion Assay

The amnion invasion assay was performed using chambers covered by human amniotic membranes as described (15, 16). Subconfluent BCE cells were labeled with 125Iodoodeoxyuridine (2,200 Ci/mmol; New England Nuclear) in low glucose Dulbecco's medium (Gibco Laboratories) for 24 h, trypsinized, and resuspended in growth medium. Cells were seeded in the upper compartment (2 cm²; 1 ml) at 1.5 x 10⁵ cell/ml. The effects of anti-bFGF IgG and rbFGF were examined by adding the reagents to the upper compartment before seeding the cells. After a 72-h incubation, the radioactivity in the amniotic membrane was counted and quantitated as the percentage of the total radioactivity. Each sample was assayed in six chambers with the sample designations masked. Experiments were carried out at least three times to confirm reproducibility.

Results

During previous studies on the expression of bFGF by endothelial cells, we observed that different clones of BCE cells, derived from separate isolations, varied in their morphology. Because bFGF expression in bovine aortic endothelial cells appeared to affect the cell phenotype (32), we characterized several of these microvessel clones in respect to properties that might relate to the levels of bFGF.

Morphological Characteristics of BCE Cell Clones

To characterize the seven BCE cell clones, we first observed their growth properties and morphology. Each clone, when split 1:4 for passage, reached confluency between 7 and 10 d after seeding. BCE-5A5, 5A2, and 8C1 cells grew ~10–20%
Figure 1. Morphological characteristics of BCE cell clones. Confluent cultures were fixed and stained with Giemsa. (A–G) Cells cultivated in aMEM supplemented with 5% calf serum; (H–J) cells treated with 10 ng/ml bFGF overnight in the same medium. Cells were photographed 3 d after confluency. (A and H) 8A2, (B) 1, (C) 5B4, (D and I) 2-0, (E) 8C1, (F and J) 5A2, and (G) SAS cells. Bar, 100 μm.
Endogenous bFGF Levels and bFGF Receptor Number

The morphological differences of the cells from different clones and their responses to exogenous rbFGF encouraged us to examine the endogenous bFGF levels of each clone. To visualize the amount of bFGF, immunoblotting was performed using polyclonal anti-bFGF antibodies (Fig. 2). Each clone displayed three major immunoreactive bands with molecular masses of 24, 22, and 18 kD in addition to a 26-kD band which represents nonspecific binding of the antibody. Preincubation of the 50 μg of antibody with rbFGF eliminated the binding to the 18-, 22-, and 24-kD bands but not the 26-kD band, thereby demonstrating the specificity of the reaction (data not shown). The lowest molecular mass band (18 kD) had the same size as purified rbFGF. The larger bFGF forms (24 and 22 kD) have been observed previously in endothelial cells (Renko, M., personal communication) and appear to represent bFGF forms extended at the amino-terminal end (2, 28, 35). Immunoblotting clearly demonstrated that 5A2 and 5A5 cells contained the highest amount of bFGF, 8A2 cells the lowest, and I, 5B4, 2-0, and 8C1 were intermediate. These data were corroborated by monitoring the ability of cell extracts from each clone to induce PA in BCE cells. This assay has been shown previously to provide a simple and quantitative assay for bFGF (22). The same rank order of bFGF levels was also observed in this assay (data not shown).

bFGF receptor binding assays with cells from the various clones showed a distribution with the 8A2 cells, having the lowest bFGF content and the highest number of receptors, and the 5A5 cells, having the highest bFGF content and the lowest number of receptors (Table I). All clones had ~7 x 10^6 low affinity binding sites per cell (data not shown). The addition of 10 ng/ml of rbFGF to 8A2, 2-0, and 5A2 cultures caused a downregulation of the number of receptors in each cell type (Table I). 8A2 cells showed the greatest degree of downregulation, while 5A2 cells showed the least. The addition of anti-bFGF IgG to the cultures did not change the binding constants for 8A2, 2-0, or 5A2 cells (data not shown). Thus, there appeared to be an inverse relationship between bFGF production and receptor number. This is in agreement with an earlier proposal by Moscatelli (20) that an inverse relationship may exist between endogenous bFGF and receptor number.

Cell Migration and Invasion Assays

Since we had shown previously that endothelial cell migration and invasion were dependent upon bFGF (16, 32), we compared cells from each of the clones with respect to these properties. The cell migration assay was performed by counting the cells that migrated into a denuded area formed by scratching a confluent monolayer with a razor blade. BCE 5A2 cells were the most migratory, while the 8A2 cells were the least (Fig. 3). The degree of migration of the other clones was roughly in the same order as observed for other properties, with 5B4 and 4 cells migrating poorly and 5A5 cells migrating well. Exogenous rbFGF stimulated the migration of cells from 5A2, 2-0 and 8A2 clones. However, the degree

Table I. Quantitation of High Affinity Binding Sites on Cells from Different Clones

| Cell clone | Sites/cell (x 10^3) after incubation with 10 ng/ml rbFGF |
|-----------|--------------------------------------------------------|
| 8A2       | 21.0                                                   |
| 2-0       | 9.0                                                    |
| 5A2       | 7.2                                                    |
| 1         | 12.0                                                   |
| 5B4       | 14.4                                                   |
| 8C1       | 13.0                                                   |
| 5A5       | 6.0                                                    |

Receptor binding assays were done as described in Materials and Methods.
Figure 3. Cell migration assay. Confluent monolayers of endothelial cells were wounded with a razor blade and incubated overnight in the absence or presence of rbFGF or anti-bFGF IgG. The number of cells that migrated into the denuded area was counted as described in Materials and Methods. The difference in cell densities in the unwounded area in seven clones was <10%. (Bars 1-3) Basal migration, migration in the presence of 10 ng/ml rbFGF, and migration in the presence of 20 μg/ml anti-bFGF IgG purified by bFGF affinity chromatography, respectively. Data illustrated are the means of eight random fields. Standard errors are shown.

The migration of 8A2 cells did not exceed the level observed with 5A2 cells. Anti-bFGF IgG inhibited the migration of all three clones. In addition, the distance that the cells migrated from the wound edge was greatest with 5A2 and 5A5 cells and least with 8A2 cells (data not shown). These data suggest that high levels of endogenous bFGF increased the distance that the cells moved as well as the number of cells that migrated.

The ability of cells from three of the clones to invade the amniotic membrane was monitored in the presence or absence of rbFGF. BCE cells normally do not penetrate the amniotic membrane as shown by Mignatti et al. (16). As expected, BCE 8A2 cells hardly invaded the membrane (Fig. 4). However, 2-0 and 5A2 cells invaded the amnion under normal conditions, with 5A2 cells being slightly more invasive. The inclusion of 10 ng/ml of rbFGF in the upper chamber potentiated the invasion by cells from each clone. However, the degree of potentiation was greatest with 8A2 cells and least with 5A2 cells. This effect was neutralized by the addition of anti-bFGF IgG (data not shown).

PA and Metalloproteinase Activities
Numerous reports have indicated that the levels of specific proteinases, such as PA and collagenase, correlate with cell invasivity (1, 14, 15, 21, 24, 30) and that endothelial cells express these proteinases (6-9, 11, 13, 16-19, 27). To establish whether proteinase levels correlated with the degree of bFGF expression, we measured the amounts of PA in cell lysates. The PA levels, as measured by the PA plate assay, varied in a manner that did not relate to the other properties (Fig. 5). Zymographic analysis and reverse fibrin zymography from the same sample indicated that differences in PA
levels were not the result of differences in PA inhibitor 1 levels (data not shown). However, the addition of 10 ng/ml rbFGF to the medium induced a prominent stimulation of PA levels in 8A2 cells, a moderate stimulation in 2-0 cells, and a poor stimulation in 5A2 cells (data not shown). This effect correlated with endogenous bFGF levels. Anti-bFGF IgG added to the cultures suppressed the level of PA activity as described earlier (32) (data not shown).

The activities of metalloproteinases were assayed by gelatin zymography (Fig. 6). The media conditioned by the various cells contained a major gelatinolytic activity with a molecular mass of 64 kD. Those clones that had high levels of the 64-kD enzyme also had minor activities with masses of 94 and 57 kD. These three bands were also seen in cell extracts but in lower amounts (data not shown). All of the gelatinolytic activities were completely blocked by the addition of 10 mM EDTA (data not shown). The molecular weights of three lytic bands are similar to or identical with previously reported gelatinolytic metalloproteinases from rabbit brain capillary endothelial cells (9). The 64-kD band may correspond to the type IV collagenase, while the 94-kD band appears similar to a gelatinase found in several cell types (9). The intensities of the 64-kD band in media correlated with the bFGF levels in the clones. 5A5 cells produced the highest amount of enzyme, while 8A2 cells produced the lowest amount. This result is consistent with the proposed requirement for type IV collagenase in endothelial cell invasion (16).

Discussion

These experiments demonstrate that a heterogeneity exists among seven clones of BCE cells with respect to endogenous bFGF levels. The results also indicate that the endogenous bFGF levels in BCE cells correlate with the cell morphology, high affinity receptor number, the degree of cell migration, the invasion of the amnion membrane, and the levels of metalloproteinase production.

BCE cells from the clone with the highest bFGF level had an elongated morphology, while cells with the lowest bFGF level retained a cobblestone morphology. Several additional reports have described a change in endothelial cell morphology upon exposure to bFGF (17, 23). These observations suggest that endogenous bFGF levels and endothelial cell morphology are closely correlated.

Cell migration and invasion also correlated directly with the level of endogenous bFGF. These responses were inversely proportional to the numbers of high affinity receptors for bFGF, indicating that receptor number may be down-regulated by the level of bFGF. The migration and invasion of the low bFGF-containing cells was increased in the presence of exogenous bFGF. However, the degree of migration and invasion never exceeded that observed with the high bFGF-producing cells, suggesting that BCE cells may have a defined limit to their response to either endogenous or exogenous bFGF.

Previously, we demonstrated that invasion of the amniotic membrane by B16 melanoma cells and BCE cells was suppressed by inhibitors and/or antibodies to PA and collagenase and that there may be linkage in activities between these two proteinases (15, 16). In the present study, the levels of metalloproteinase activities were found to correlate with bFGF levels and, thus, with the invasivity of the cells; however, the PA activities did not. This agrees with our previous data, suggesting metalloproteinases as the final step in a cascade of proteolytic activations required for invasion (11, 15). The lack of a correlation between PA levels and the degree of invasion may indicate that, once a minimal level of PA production is achieved to insure sufficient activation of procollagenase, further increases in PA activity are of little consequence. Alternatively, a recent report by Ossowski (26) has shown that only receptor-bound urokinase-type PA is critical for invasion. Therefore, it is possible that the amount of receptor-bound urokinase-type PA in these BCE cells may correlate with bFGF concentrations. At present, it is not possible to measure this parameter for the different BCE clones since pure bovine urokinase-type PA is not available and receptor binding displays a species specificity.

In these experiments, some of the BCE clones used were isolated from individual bovine adrenal cortices. We have no idea of the precise vascular origin of these cells other than that they are from the adrenal cortex microvasculature. Our isolation procedure does not permit us to differentiate between true capillary endothelial cells and cells from postcapillary venules or small arterioles. It is interesting to speculate, however, whether within a single microvessel endothelial cells vary in properties such as bFGF production and whether this may relate to unique functional roles during processes such as angiogenesis. Thus, only certain cells may initiate capillary invasion, while others may simply divide to provide the additional cells required for the elongating vessel.

Since cells with high levels of bFGF may have a growth advantage compared with cells producing low levels of bFGF, the high levels of bFGF found in many cultured cells may represent a tissue culture selection artifact as these cells would be easier to establish in culture. Thus, in vivo bFGF levels may be significantly lower than those observed in vitro. A potential biological advantage of this state is that bFGF production could be induced by specific agents under the appropriate conditions. The 8A2 cells may provide a cell strain to assay for such agents that induce bFGF synthesis.

Figure 6. Metalloproteinase zymography. Nonconcentrated conditioned media were assayed for metalloproteinases as described in Materials and Methods. The gel was incubated in 2 mM CaCl₂, 50 mM Tris-HCl buffer (pH 7.6). (Lanes 1-7) 8A2, 1-0, 8C1, 5B4, 5A2, and 5A5 cells, respectively.

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