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Basic Fibroblast Growth Factor Modulates Integrin Expression in Microvascular Endothelial Cells

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During angiogenesis capillary endothelial cells undergo a coordinated set of modifications in their interactions with extracellular matrix components. In this study we have investigated the effect of the prototypical angiogenic factor basic fibroblast growth factor (bFGF) on the expression and function of several integrins in microvascular endothelial cells. Immunoprecipitation experiments with antibodies to individual subunits indicated that microvascular cells express at their surface several integrins. These include the $\alpha1\beta1$, $\alpha2\beta1$, and $\alpha3\beta1$ laminin/collagen receptors; the $\alpha6\beta1$ laminin receptor; the $\alpha5\beta1$ and $\alpha v\beta1$ fibronectin receptors; the $\alpha6\beta4$ basement membrane receptor; and the $\alpha v\beta3$ and $\alpha v\beta5$ vitronectin receptors. Treatment with bFGF caused a significant increase in the surface expression of the $\alpha2\beta1$, $\alpha3\beta1$, $\alpha5\beta1$, $\alpha6\beta1$, $\alpha6\beta4$, and $\alpha v\beta5$ integrins. In contrast, the level of expression of the $\alpha1\beta1$ and $\alpha v\beta3$ integrins was decreased in bFGF-treated cells. Immunoprecipitation of metabolically labeled cells indicated that bFGF increases the biosynthesis of the $\alpha3$, $\alpha5$, $\alpha6$, $\beta4$, and $\beta5$ subunits and decreases the production of the αv and $\beta3$ subunits. These results suggest that bFGF modulates integrin expression by altering the biosynthesis of individual α or β subunits. In accordance with the upregulation of several integrins observed in bFGF-treated cells, these cells adhered better to fibronectin, laminin, vitronectin, and type I collagen than did untreated cells. The largest differences in $\beta1$ integrin expression occurred ~72 h after exposure to bFGF, at a time when the expression of the endothelial cell-to-cell adhesion molecule endoCAM was also significantly upregulated. In contrast, a shorter exposure to bFGF (24–48 h) was required for the maximal induction of plasminogen activator production in the same cells. Taken together, these results show that bFGF causes significant changes in the level of expression and function of several integrins in microvascular endothelial cells.

INTRODUCTION

Angiogenesis is a complex process regulated by the interaction of capillary endothelium with growth factors and extracellular matrix (ECM)[1] proteins (Folkman and Klagsbrun, 1987; Ingber and Folkman, 1989b; Rifkin and Moscatelli, 1989). Basic fibroblast growth factor (bFGF) is a heparan sulfate–binding growth factor that is angiogenic in vivo (Folkman and Klagsbrun, 1987) and induces an "angiogenic phenotype" consisting of increases in protease production, motility, and cell proliferation in cultured endothelial cells (Moscatelli *et al.*,

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[1] Abbreviations used: BCE cells, bovine capillary endothelial cells; bFGF, basic fibroblast growth factor; COL I, type I collagen; ECM, extracellular matrix; endoCAM, endothelial cell adhesion molecule; FN, fibronectin; LM, laminin; MEM, minimal essential medium; PA, plasminogen activator; PBS, phosphate buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; VN, vitronectin.

1986; Presta *et al.*, 1986). The regulatory signals that determine whether endothelial cells grow, branch, differentiate, or involute in response to bFGF have been shown to be provided, in part, by the local tissue microenvironment, defined by the interaction of the cell with ECM components (discussed in Ingber and Folkman, 1989b).

The ECM is thought to play at least two roles during bFGF-induced angiogenesis. First, ECM can directly affect cell behavior by binding to distinct types of cell surface adhesion receptors, such as the integrin family of receptors (Buck and Horwitz, 1987; Ruoslahti and Pierschbacher, 1987; Albelda and Buck, 1990; Hemler, 1990; Springer, 1990; Ginsberg *et al.*, 1992; Hynes, 1992). Second, bFGF binds to heparan sulfate proteoglycans in the ECM and this interaction may potentiate the biological activity of bFGF (Flaumenhaft *et al.*, 1989; Presta *et al.*, 1989; Yayon *et al.*, 1991; Olwin and Rapraeger, 1992). The response of endothelial cells to growth factors can be influenced by the integrity and composition of the ECM as well as the adhesive capacity of the cells (Gospodarowicz *et al.*, 1978; Ingber *et al.*, 1986, 1987; Ingber and Folkman, 1988, 1989a). However, the mechanisms by which ECM proteins, adhesion receptors and soluble growth factors interact during angiogenesis remain unknown.

Large-vessel and microvascular endothelial cells express a number of integrins that function in adhesion to the ECM (Albelda *et al.*, 1989; Cheng and Kramer, 1989; Basson *et al.*, 1990; Kramer *et al.*, 1990). Integrins are heterodimeric receptors composed of an α and a β subunit. At present we know of at least 8 different β and 15 different α subunits that can variously combine to form 21 receptors with distinct ligand specificity. The processes of endothelial cell migration, adhesion, and tube formation that occur during angiogenesis may depend on integrin activity. Integrins are required for endothelial cell migration on various substrata (Bauer *et al.*, 1992b; Leavesley *et al.*, 1992, 1993). In addition, there is evidence that endothelial cell tube formation in vitro is blocked by monoclonal antibodies to the $\alpha 6$ and $\beta 1$ subunits (Bauer *et al.*, 1992a). Finally, a limited survey showed that transforming growth factor β (TGFβ) and bFGF can modulate the $\alpha 5\beta 1$ and $\alpha 2\beta 1$ integrins in human endothelial cells (Enenstein *et al.*, 1992). In this study, we have determined the repertoire of integrins expressed by bovine microvascular endothelial cells and investigated the capacity of bFGF to modulate their levels of expression. The results indicate that BCE cells express at least nine different integrins and that bFGF regulates the expression of these integrins both positively and negatively. The resulting changes in adhesion may be part of the complex modification of endothelial cell behavior that occurs following exposure to an angiogenic stimulus.

MATERIALS AND METHODS

Cell Cultures

Microvascular endothelial cells were isolated from the bovine adrenal cortex as previously described (Folkman *et al.*, 1979) and maintained in alpha minimum essential medium (MEM) supplemented with 5% fetal calf serum (Intergen, Purchase, NY) and 2 mM L-glutamine (Irvine Scientific, Santa Ana, CA). All experiments were performed with cultures between passages 12 and 19.

Antibodies

The anti-integrin antibodies used in this study were raised by immunization with synthetic peptides reproducing C-terminal portions of individual integrin subunits. The cytoplasmic peptide antibodies to $\alpha 1$ (Tarone *et al.*, 1993), $\alpha 2$ (Defilippi *et al.*, 1992), $\alpha 5$ and $\beta 1$ (Giancotti and Ruoslahti, 1990), $\alpha 6$ and $\beta 4$ (Giancotti *et al.*, 1992), and αv, $\beta 3$, and $\beta 5$ (Vogel *et al.*, 1993) were previously described. The $\alpha 3$ subunit–specific polyclonal antibody was elicited by immunization of a rabbit with a 34-mer synthetic peptide reproducing the cytoplasmic domain of the chicken $\alpha 3$ subunit (KKRRASTGAMYEAKGQKAEMRIQPSETERLTDDY). For the time course experiment, we used a previously described polyclonal antiserum that reacts with high affinity to $\beta 1$ and with lower affinity to $\beta 3$ (Buck and Horwitz, 1987). All of the polyclonal antibodies used cross-react with bovine capillary endothelial (BCE) cells. Endothelial cell adhesion molecule (EndoCAM) was detected by using a previously described monoclonal antibody (Albelda *et al.*, 1990).

Cell Labeling and Immunoprecipitation

BCE cells were plated at subconfluence in 15-cm dishes (Falcon, Becton Dickinson, Lincoln Park, NJ). Cells were incubated in fresh alpha MEM-5% fetal calf serum in the presence or absence of 30 ng/ml of human recombinant bFGF (Synergen, Boulder, CO). After 48 h, cells to be surface labeled were washed with phosphate-buffered saline (PBS) and detached with 0.1% collagenase (Worthington, Freehold, NJ), 0.01% EDTA, and 0.25% bovine serum albumin (Sigma, St. Louis, MO) at 37°C. The suspended cells were washed three times with PBS, and surface proteins were labeled with 158 μg/ml lactoperoxidase (Sigma), 0.0038% H_2O_2, and $Na^{125}I$ (1 mCi/ml) (New England Nuclear, Wilmington, DE). Cells were washed three times with alpha MEM containing NaN_3 and solubilized in 25 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.5% Triton X-100 containing leupeptin (10 μg/ml) (Sigma), pepstatin A (4 μg/ml) (Sigma), and aprotinin (50 μg/ml) (Sigma).

For metabolic labeling, cells treated or untreated with bFGF as specified above were preincubated at 37°C for 1 h with met/cys-free MEM supplemented with 5% dialyzed fetal calf serum and then incubated at 37°C for 3 h in met/cys-free MEM supplemented with 5% dialyzed fetal calf serum and 100 μCi/ml ^{35}S Trans label (New England Nuclear). Cells were washed with cold PBS, extracted for 20 min with cold 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, 150 mM NaCl, and 50 mM Tris-HCl, pH 7.4 (RIPA buffer) containing protease inhibitors and removed from the dish with a Costar cell scraper. The ^{125}I- and ^{35}S-labeled cell lysates were clarified in an Eppendorf microfuge for 30 min and preadsorbed with normal rabbit immunoglobulin (Ig) Gs and protein A-Sepharose (Sigma) for 1 h at 4°C. To exclude the possibility that changes in integrin expression were due to an effect of bFGF on cell proliferation (i.e., increase in cell number), all the labeled cell extracts were normalized according to trichloroacetic acid–precipitable radioactivity. Saturating amounts of the various antibodies were added to aliquots of the cell lysate and rotated for 1 h at 4°C. Protein A-Sepharose beads were added to each sample and rotated for 1 h at 4°C. The resin was washed five times with RIPA buffer. The beads were boiled for 5 min in nonreducing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and electrophoresed on 7% SDS-poly-

acrylamide gels. Gels were dried and exposed on XAR-5 film (Eastman Kodak, Rochester, NY). Protein band intensity was scanned by PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Adhesion Assay

Tissue culture plates (96 well) (Falcon, Becton Dickinson) were coated for 1 h with 75 μl of PBS containing fibronectin (FN) (bovine plasma derived, GIBCO, Gaithersburg, MD), vitronectin (VN) (bovine plasma derived, Telios, San Diego, CA), laminin (LM) (Engelbreth-Holm-Swarm sarcoma derived, GIBCO), and type I collagen (COL I) (bovine plasma derived, Collaborative Research, Bedford, MA) at the indicated concentrations. The plates were blocked with 0.05% bovine serum albumin in PBS. To avoid synthesis and secretion of adhesive proteins during the assay, cells grown in the presence or absence of bFGF were treated with 20 μM cycloheximide for 1 h and 1 μM monensin for 10 min before the assay. Cells were washed and resuspended (10^6 cells/ml) in alpha MEM. Fifty microliters of this cell suspension was added to the coated wells, and the cells were allowed to attach to the substrata for 1 h at 37°C. At the end of this time, unattached cells were removed by washing the plates twice with PBS. The attached cells were fixed with 3.7% paraformaldehyde for 20 min and washed. Cells were permeabilized with 20% methanol and stained with 0.5% crystal violet in 20% methanol. The stain was eluted with 0.1 M sodium citrate, pH 4.2, in 50% ethanol, and the absorbancy was measured at 550 nm. Using this method of crystal violet staining, there is a linear relationship between cell number and optical density as previously described (Kueng *et al.*, 1989).

PA Assay

BCE cells in 35-mm dishes were treated with 30 ng/ml bFGF in 5% calf serum. Medium containing bFGF was changed every day. To prepare cell extracts, cells were washed twice with PBS and extracted with 0.5% Triton X-100 in 0.1 M Tris-HCl, pH 8.1. Aliquots of the cell extracts (2 μg protein) were assayed on ^{125}I-labeled fibrin-coated plates as described previously (Gross *et al.*, 1982).

RESULTS

BCE Cells Express β1, β3, β4, and β5 Integrin Receptors

The repertoire of integrins expressed by BCE cells was determined by surface labeling and immunoprecipitation experiments. Rabbit antibodies to synthetic peptides reproducing the cytoplasmic domains of human integrin subunits were used in these experiments inasmuch as these antibodies generally cross-react with all mammalian integrins. As shown in Figure 1, the β1 cytoplasmic peptide antibody immunoprecipitated the β1 subunit associated with α subunits migrating as a broad band of ~150 kDa and with one α subunit migrating at 190 kDa. In agreement with this result, immunoprecipitation with antibodies to individual α subunits indicated that BCE cells express at their surface several distinct β1 integrins including α1β1, α5β1, α3β1, and α6β1 integrins. In addition to α6 and β1, the α6 cytoplasmic peptide antibody immunoprecipitated a band of ~200 kDa. Because a band of identical molecular weight was immunoprecipitated together with α6 by the β4 cytoplasmic peptide serum (not shown), we conclude that BCE cells also express the α6β4 integrin. The α2β1 integrin was not detected in unstimulated BCE cells.

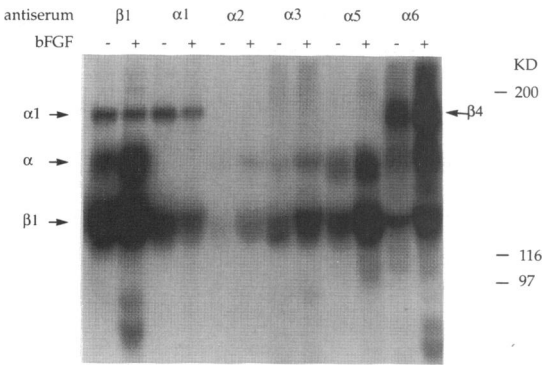

Figure 1. Effect of bFGF on β1 integrin expression in BCE cells. Subconfluent cultures were incubated for 48 h in the absence or in the presence of 30 ng/ml bFGF. Cell suspensions were surface-labeled with ^{125}I and extracts were immunoprecipitated with β1, α1, α2, α3, α5, or α6 cytoplasmic peptide antibodies. Samples were boiled in nonreducing sample buffer and analyzed by SDS-PAGE. Scanning analysis of protein bands was performed by PhosphorImager.

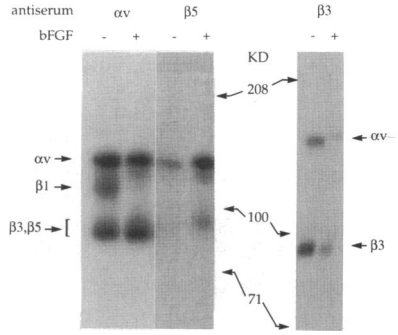

Figure 2. Effect of bFGF on expression of αv subunit–containing integrins in BCE cells. Subconfluent cultures were incubated for 48 h in the absence or in the presence of 30 ng/ml bFGF. Cell suspensions were surface-labeled with ^{125}I and extracts were immunoprecipitated with αv, β5, or β3 cytoplasmic peptide antibodies. Samples were boiled in nonreducing sample buffer and analyzed by SDS-PAGE.

The expression by BCE cells of integrins containing an αv subunit was examined by performing immunoprecipitations with antibodies reacting with αv, β3, and β5 (Figure 2). The αv cytoplasmic peptide antibody immunoprecipitated complexes of αv with a β subunit comigrating with β1 and with β subunits of lower molecular weight. Immunoprecipitation with antibodies to β3 and β5 peptides indicated that these lower molecular weight β subunits consisted mostly of β3 and smaller amounts of β5. These results indicate that BCE cells express the α1β1 and α3β1 collagen/LN receptors, the α6β1 LN receptor, the α5β1 and αvβ1 FN receptors, the α6β4 basement membrane receptor, and the αvβ3 and αvβ5 VN receptors.

Effect of bFGF on Integrin Receptors in BCE Cells

To evaluate the effect of bFGF on integrin expression in BCE cells, we compared the integrins expressed at

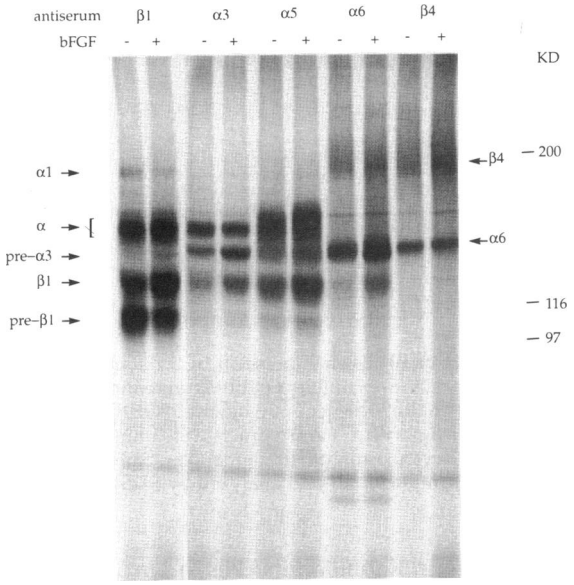

Figure 3. Effect of bFGF on $\beta 1$ and $\beta 4$ integrin biosynthesis in BCE cells. Subconfluent cultures were incubated for 48 h in the absence or in the presence of 30 ng/ml bFGF. Cell suspensions were labeled with ^{35}S met/cys during the final 3 h and immunoprecipitated with $\beta 1$, $\alpha 3$, $\alpha 5$, $\alpha 6$, or $\beta 4$ cytoplasmic peptide antibodies. Samples were boiled in nonreducing sample buffer and analyzed by SDS-PAGE.

the surface of cells treated with 30 ng/ml bFGF for 48 h with those of untreated cells. Immunoprecipitation of surface-labeled cells with integrin antibodies indicated that bFGF causes significant changes in the level of expression of several integrins (Figure 1). With the exception of $\alpha 1\beta 1$, which was decreased in bFGF-treated cells, all the other $\beta 1$ integrins were expressed at higher levels in bFGF-treated cells than in untreated cells. Scanning analysis by PhosphorImager of the protein bands after SDS-PAGE indicated that the decrease of $\alpha 1$ was approximately twofold, whereas the increases of $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 5\beta 1$, and $\alpha 6\beta 1$ were between two- and fourfold. Immunoprecipitation with both $\alpha 6$ and $\beta 4$ antibodies indicated that the $\alpha 6\beta 4$ integrin was also expressed at higher levels (approximately fourfold increase) in bFGF-treated than in untreated cells.

Figure 2 also shows the results of the comparison of αv-containing integrins in bFGF-treated and -untreated cells. Immunoprecipitation with αv cytoplasmic peptide antibodies indicated that the $\alpha v\beta 1$ complex almost completely disappeared from the cell surface upon bFGF treatment. No other changes in the level of expression of αv integrins upon bFGF treatment could be detected by using antibodies to αv. However, immunoprecipitation with $\beta 3$ and $\beta 5$ antibodies indicated a decreased expression of $\alpha v\beta 3$ and an increased expression of $\alpha v\beta 5$ in bFGF-treated cells. Thus, bFGF treatment increases the expression of $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 5\beta 1$, $\alpha 6\beta 1$, $\alpha 6\beta 4$, and $\alpha v\beta 5$, and decreases the expression of $\alpha 1\beta 1$ and $\alpha v\beta 3$ on the cell surface of BCE cells.

To assess whether the changes in surface-labeled integrins induced by bFGF were due to changes in the rate of biosynthesis or turnover of individual subunits, cells treated with 30 ng/ml bFGF for 48 h or left untreated were metabolically labeled for 3 h with 100 μCi/ml ^{35}S met/cys and immunoprecipitated with integrin antibodies. Immunoprecipitation with $\beta 1$ cytoplasmic peptide antibodies indicated that the total $\beta 1$ subunit synthesized over the labeling period by bFGF-treated cells was not substantially different compared with untreated cells (Figure 3). However, there was more $\beta 1$ subunit recovered in mature form than in precursor form in the bFGF-treated cells, whereas the opposite was true for untreated cells. This suggests that the $\beta 1$ subunit is processed at a faster rate upon bFGF treatment. However, a pulse-chase experiment would be required to demonstrate differences in the rate of processing. Analysis of the biosynthesis of $\alpha 3$, $\alpha 5$, and $\alpha 6$ indicated a higher rate of synthesis of all these subunits in bFGF-treated than in -untreated cells with the largest changes observed for $\alpha 5$ and $\alpha 6$. The data suggests that the higher expression of $\beta 1$ integrins at the surface of bFGF-treated cells is largely due to the increased biosynthesis of individual α subunits. The increased rate of processing of $\beta 1$ is consistent with this hypothesis, as only paired α and β subunits undergo oligosaccharide processing and transport from the endoplasmic reticulum to the Golgi apparatus (Cheresh and Harper, 1987; Heino *et al.*, 1989).

The synthesis of $\beta 4$ was found to be slightly increased as measured by scanning analysis of protein bands (Figure 3). Immunoprecipitation with αv, $\beta 3$, and $\beta 5$ antibodies indicated a slightly decreased biosynthesis of αv, a marked decrease of $\beta 3$, and a marked increase of $\beta 5$ (Figure 4). These results suggest that the decreased expression of $\alpha v\beta 3$ and the increased expression of $\alpha v\beta 5$ at the cell surface are due to the combined effects of decreased biosynthesis of αv and a switch in β chain synthesis and usage. The almost complete disappearance of $\alpha v\beta 1$ from the surface of bFGF-treated cells

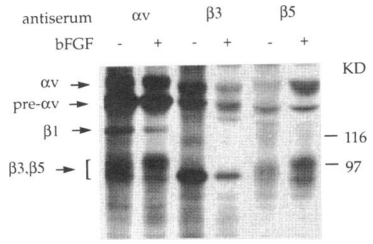

Figure 4. Effect of bFGF on biosynthesis of vitronectin receptors in BCE cells. Subconfluent cultures were incubated for 48 h in the absence or in the presence of 30 ng/ml bFGF. Cell suspensions were labeled with ^{35}S met/cys during the final 3 h and extracts were immunoprecipitated with αv, $\beta 3$, or $\beta 5$ cytoplasmic peptide antibodies. Samples were boiled in nonreducing sample buffer and analyzed by SDS-PAGE.

may instead be attributed to the increased biosynthesis of α subunits associating with $\beta 1$ in these cells.

Increased Substrate Adhesion of bFGF-treated BCE Cells

Because the integrins affected by bFGF include receptors for collagens, FN, LN, and VN, adhesion assays were performed to measure the ability of bFGF-treated BCE cells to interact with these matrix proteins. Control cells and cells treated with bFGF for 48 h were plated on culture dishes coated with increasing concentrations of COL I, FN, LM, or VN. As shown in Figure 5, control cells adhered to all the substrates in a dose-dependent way. bFGF-treated cells showed a significant increase in adhesion to all the substrata when compared with untreated cells. Direct microscopic observation of adherent cells indicated that bFGF-treated cells spread more than untreated cells when plated on dishes coated with the same amounts of the different ECM proteins (Figure 6). In addition, the treated cells were distinctly more spindle shaped than their nontreated counterparts on COL I, FN, and LM, but not on VN. In conclusion, the changes in the expression of different integrins on the surface of bFGF-treated BCE cells were paralleled by an increased capacity of these cells to adhere to various matrix proteins.

Kinetics of Cell Surface $\beta 1$ Integrin Expression by bFGF

To evaluate the changes in the level of expression of integrins in the context of the other changes induced by bFGF in BCE cells, we compared the kinetics of expression of $\beta 1$ integrins to the kinetics of induction of plasminogen activator (PA) production, representing

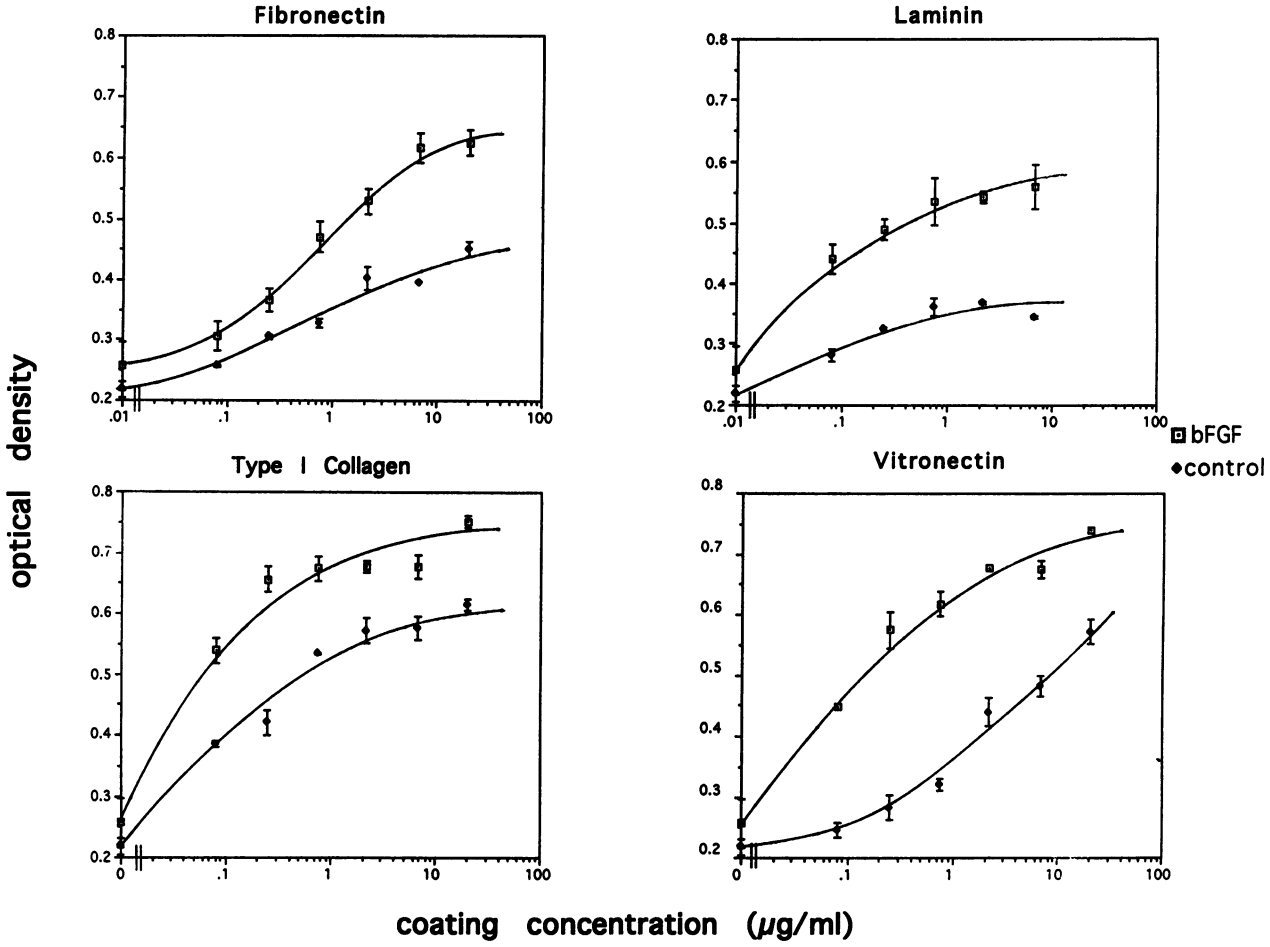

Figure 5. Effect of bFGF on adhesion of BCE cells to various substrates. BCE cells were incubated for 48 h in the absence or in the presence of 30 ng/ml bFGF. Cultures were treated with cycloheximide (1 h) and monensin (10 min). Cells were added in serum free medium to wells coated with different concentrations of FN, LM, COL I, or VN. Cells were allowed to attach to the various substrates for 1 hr at 37°C. Adherent cells were fixed and stained with crystal violet. The number of cells was determined by measuring optical density as described in MATERIALS AND METHODS.

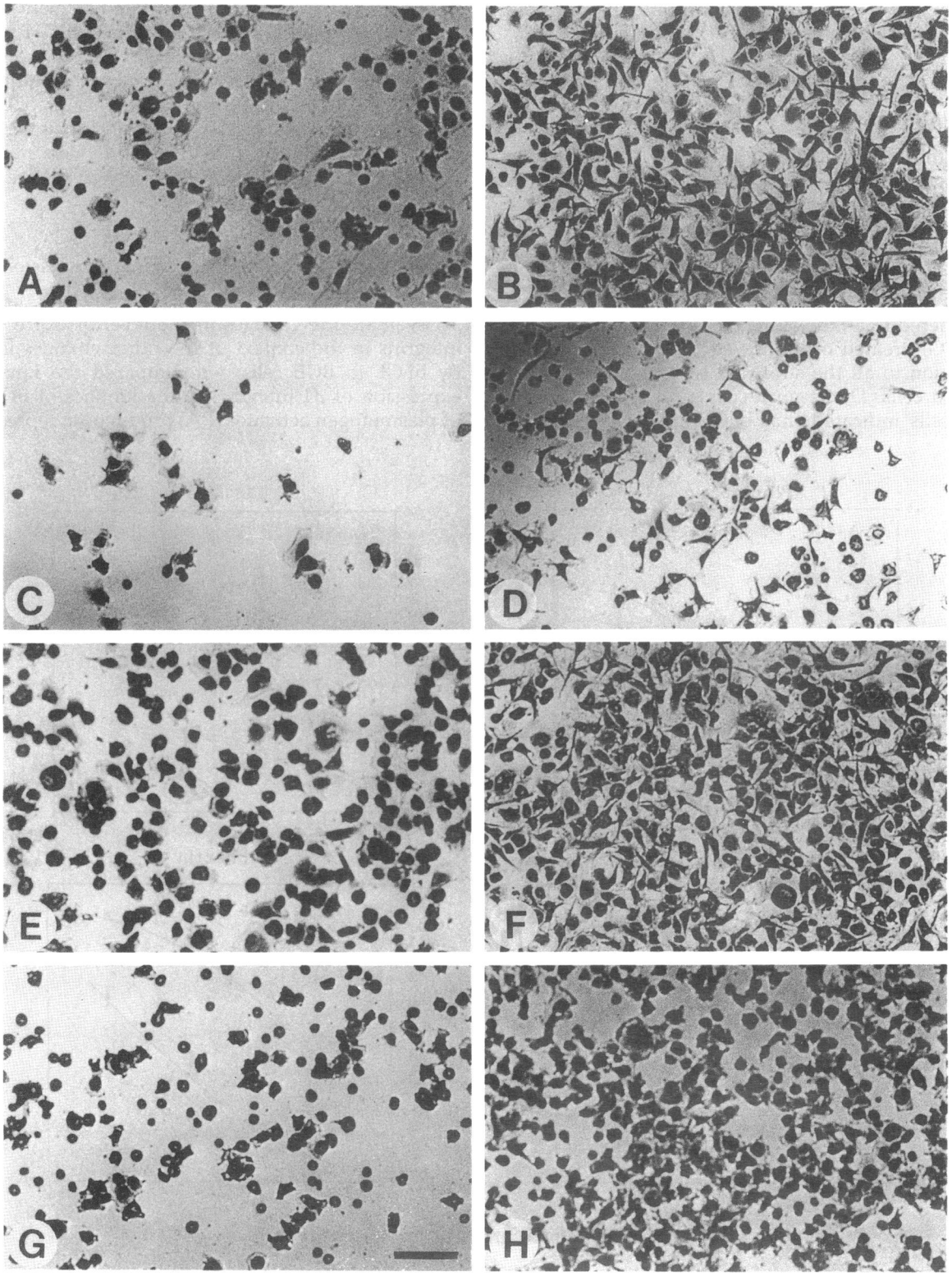

Figure 6. Micrographs of bFGF-treated BCE cells on different substrates. BCE cells were incubated for 48 h in the absence (A, C, E, and G) or in the presence of 30 ng/ml bFGF (B, D, F, and H). Cultures were treated with cycloheximide (1 h) and monensin (10 min). Cells were added in serum free medium to wells coated with FN (A and B), LM (C and D), COL I (E and F), or VN (G and H). Cells were allowed to attach to the various substrates for 1 h at 37°C. Adherent cells were fixed and stained with crystal violet. Bar, 80 μm.

changes in the early invasive phase of angiogenesis. As endoCAM has been hypothesized to be involved in the process of cell-cell interaction during angiogenesis (Albelda *et al.*, 1990), a possible effect of bFGF on the quantity of this protein on the cell surface was also examined. BCE cells were treated with 30 ng/ml bFGF for 96 h, and the cultures were analyzed each day for surface-labeled β1 integrins, surface-labeled endoCAM, and cell-associated PA activity. The results showed that bFGF induces an increase in the levels of β1 integrins, endoCAM, and PA activity, but with different kinetics (Figure 7A and B). In agreement with the timing of the events occurring during angiogenesis, PA production was the first event that reached its maximum expression in bFGF-treated cells. Maximal PA activity was detected after 24–48 h of treatment with the growth factor (Figure 7B). Cell surface appearance of β1 integrins and endoCAM were stimulated by bFGF with slower kinetics. Indeed, when the two molecules were quantitated by counting the corresponding bands excised from the gel after autoradiography, both β1 integrin receptor and endoCAM showed a maximal increase after 72 h of treatment with the growth factor (Figure 7B). Thus, the general increase of β1 integrins induced by bFGF follows the increase in PA activity, but is cotemporary to the upregulation of endoCAM.

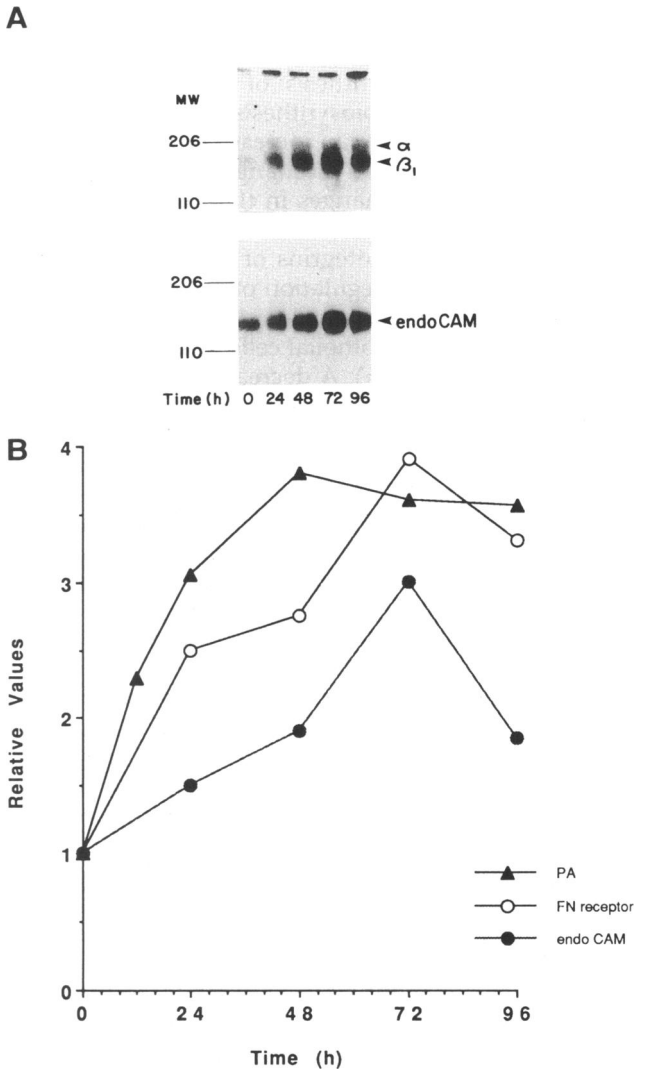

Figure 7. Kinetics of induction of β1 integrin expression, endoCAM expression, and PA production. BCE cells were treated with 30 ng/ml bFGF for 96 h. Each day cultures were surface-labeled with ^{125}I. Cell lysates were immunoprecipitated with anti-β1 antibody or anti-endoCAM antibody and immunocomplexes were analyzed by SDS-PAGE under non-reducing conditions (A). After autoradiography, the bands corresponding to β1 integrin subunit (○) and to endoCAM (●) were excised from the gel and counted in a γ-counter (B). Unlabeled cultures were assayed for cell-associated PA activity (▲) on ^{125}I-labeled fibrin-coated plates (B). Data shown is from a single experiment that is representative of the experiment performed twice.

DISCUSSION

The interaction of microvascular endothelial cells with adjacent extracellular matrices changes dramatically during angiogenesis. Before angiogenesis, the microvascular endothelial cells are in a quiescent state, adherent to adjacent cells and to a stable basement membrane ECM. Upon stimulation by angiogenic factors, endothelial cell sprouts hydrolyze and penetrate the basement membrane leading to subsequent cell migration into the interstitium toward the source of the angiogenic factor. This invasive and proliferative phase of angiogenesis is followed by a differentiative phase in which endothelial cells remodel the underlying ECM by secreting new matrix components. Cell-cell interactions are re-established and the endothelial cells return to a quiescent state. Endothelial cells, therefore, interact with different basement membrane and interstitial molecules during the various phases of angiogenesis. These interactions are mediated by surface molecules interacting with the extracellular environment and transmitting information across the membrane to the interior of the cell. A family of molecules important in such processes is the integrins. We have, therefore, examined the surface expression of integrins on BCE cells after exposure to bFGF, a known angiogenic factor.

Our results indicate that bFGF is both a positive and negative regulator of integrin expression in microvascular cells. Exposure of BCE cells to bFGF increases the levels of expression of different β1 integrins including $\alpha2\beta1$, $\alpha3\beta1$, $\alpha5\beta1$, and $\alpha6\beta1$. bFGF also increases the expression of $\alpha6\beta4$ and $\alpha v\beta5$. In contrast, the expression of $\alpha1\beta1$, $\alpha v\beta1$, and $\alpha v\beta3$ is decreased by bFGF. Metabolic labeling of bFGF-treated BCE cells followed by immunoprecipitation with anti-integrin antibodies has shown that for most of the integrin heterodimers investigated, the modulation of their expression at the cell surface reflects concomitant modification of the biosynthesis of at least one of the two composing sub-

units. In particular, bFGF induces the biosynthesis of $\alpha 2$ and increases that of $\alpha 3$, $\alpha 5$, $\alpha 6$, $\beta 4$, and $\beta 5$, whereas it decreases the biosynthesis of αv and $\beta 3$. These changes in the rates of biosynthesis of integrin subunits may be a result of either an increase or decrease in the rate of their transcription or changes in the stability of their mRNAs. Thus, changes in the rates of synthesis of integrin subunits can account for the differences in expression of various integrins of BCE cell surface. A similar mechanism of regulation of cell surface expression of different integrin heterodimers has been described for human endothelial cells treated with tumor necrosis factor α (TNFα). A decrease of the expression of $\alpha v\beta 3$ is achieved in these cells by a decrease in synthesis of the $\beta 3$ subunit, but not of the αv subunit (Defilippi *et al.*, 1991b), whereas the modulation of $\alpha 6\beta 1$ and $\alpha 1\beta 1$ occurs via an alteration of the synthesis of α subunits, but not of the $\beta 1$ subunit (Defilippi *et al.*, 1991a, 1992). The largest changes in the expression of integrins induced by bFGF include the upregulation of the collagen/laminin receptors $\alpha 2\beta 1$ and $\alpha 3\beta 1$ and of the fibronectin receptor $\alpha 5\beta 1$. In accord with these results, we have observed that treatment with bFGF results in an increase in the adhesion and spreading of BCE cells on FN, LM, and COL I. bFGF-treated cells also showed an increased capacity to adhere and spread on VN-coated substrata. Because increase of the expression on the cell surface of the VN receptor $\alpha v\beta 5$ was paralleled by a decrease of the VN receptor $\alpha v\beta 3$, this suggests that $\alpha v\beta 5$ is more crucial than $\alpha v\beta 3$ in mediating the adhesion of BCE cells to VN. However, the determination of the relative importance of each of these VN receptors would require the availability of specific monoclonal antibodies to each integrin type.

An interesting observation in this study is that microvascular endothelial cells express $\alpha 6\beta 4$ basement membrane receptor and that this integrin is upregulated upon bFGF exposure of the cells. Although previous studies using immunohistochemical methods have detected the $\beta 4$ subunit in some blood vessels (Carter *et al.*, 1990; Ryynanen *et al.*, 1991; Kennel *et al.*, 1992), until now a biochemical demonstration of the presence of $\alpha 6\beta 4$ in endothelial cells has not been provided. In fact, an in vitro analysis of human umbilical vein endothelial cells showed that these cells do not express $\alpha 6\beta 4$ (Defilippi *et al.*, 1992). Our results demonstrating the upregulation of $\alpha 6\beta 4$ on microvascular cells treated with bFGF suggest that the previously observed variable staining of $\alpha 6\beta 4$ in blood vessels may reflect the presence of the receptor on microvascular endothelial cells undergoing mitoses and/or angiogenesis.

In several instances, we observed that bFGF upregulates one integrin, while downregulating a second integrin that binds to the same ligand. The capacity of a cytokine to modulate in opposite directions the expression of different integrin receptors that bind to the same ligand is not unusual. Indeed, TNFα has been shown to affect differently the expression of LM receptors $\alpha 6\beta 1$ and $\alpha 1\beta 1$ on human umbilical vein endothelial cells (Defilippi *et al.*, 1991a, 1992). Interestingly, these same endothelial cells have been shown to respond to TNFα and interferon γ (IFNγ) with a decrease of $\alpha v\beta 3$, whereas the expression of $\alpha 2\beta 1$, $\alpha 3\beta 1$, and $\alpha 5\beta 1$ was not affected by either TNFα, interleukin 1β (IL-1β), or IFNγ (Defilippi *et al.*, 1991b, 1992). Integrins that bind the same ligand but mediate distinct biological functions have been described previously. For instance, both $\alpha 5\beta 1$ and $\alpha v\beta 1$ integrins mediate the binding and adhesion of cells to fibronectin, but only $\alpha 5\beta 1$ can promote matrix-assembly of fibronectin (Zhang *et al.*, 1993). There is evidence suggesting that the different biological functions exerted by integrins binding to the same ligand may depend on the structure of their cytoplasmic domains. Chimeric integrins expressing the $\alpha 2$ extracellular domain and cytoplasmic domain of either $\alpha 2$, $\alpha 5$, or $\alpha 4$ show comparable avidity for collagen and laminin. However, the $\alpha 4$ cytoplasmic domain mediates migration on collagen and laminin whereas $\alpha 2$ and $\alpha 5$ cytoplasmic domains do not. In contrast, $\alpha 2$ and $\alpha 5$ mediate collagen gel contraction whereas $\alpha 4$ does not (Chan *et al.*, 1992). On the basis of all these results, it is possible that some of the changes in the repertoire of integrins expressed by microvascular cells induced by bFGF may enable these cells to respond differently than untreated cells to extracellular matrices of the same composition.

A 72-h exposure to bFGF was required for maximal induction of expression of $\beta 1$ integrins (Figure 7). The increase of $\beta 1$ integrin expression appears, therefore, to represent a late response of cultured endothelial cells to an angiogenic factor. This response could be related to the late differentiative phase that occurs in vivo during neovascularization. This is consistent with the finding that a similar time course of induction by bFGF is observed for endoCAM, a membrane protein thought to be involved in endothelial cell-cell interactions (Albelda *et al.*, 1990). Shorter exposures to bFGF result in the induction of endothelial cell motility (2–4 h), proliferation (10–12 h), and protease production (24 h): events related to the early invasive phase of angiogenesis (Mignatti *et al.*, 1989). Similarly, in human umbilical vein endothelial cells treated with TNFα, alterations of protease production and cell-matrix and cell-cell adhesion follow different kinetics (Defilippi *et al.*, 1992). We have not analyzed the kinetics of induction or downregulation of each integrin affected by bFGF. It appears that some of the changes in integrin expression observed in bFGF-treated cells occur earlier than 72 h. Capillaries, in situ, normally express relatively low levels of many of the integrins that bind to the ECM components likely to be enriched in areas where angiogenesis occurs (Albelda, 1991). It is possible that factors such as bFGF upregulate the expression of some integrins earlier than others, thus promoting capillary mi-

gration. Consistent with this hypothesis is the observation that bFGF-treated microvascular cells acquire a migratory morphology when plated on several ECM components, especially LM.

The results described in this paper should also be considered in light of the observation that the modification of the adhesive capacity of the ECM influences the response of endothelial cells to soluble growth factors (Ingber *et al.*, 1987). In particular, endothelial cells grow or differentiate in response to bFGF as a function of the adhesivity or mechanical integrity of the ECM (Ingber and Folkman, 1989a). Our results suggest that the response of endothelial cells to bFGF and possibly other growth factors, can change during long-term exposure, perhaps as a result of alterations in the ECM. This can lead to the expression of different adhesive molecules on the cell surface to accommodate changes in the ECM. Thus, bFGF appears to cause a coordinated set of changes in endothelial cells that may explain some of the dramatic changes occurring in endothelial cells during angiogenesis.

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