VASCULAR ENDOTHELIAL CELLS MAINTAINED IN THE ABSENCE OF FIBROBLAST GROWTH FACTOR UNDERGO STRUCTURAL AND FUNCTIONAL ALTERATIONS THAT ARE INCOMPATIBLE WITH THEIR IN VIVO DIFFERENTIATED PROPERTIES

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ABSTRACT

Vascular endothelial cells cultured in the presence of fibroblast growth factor (FGF) adopt at confluence a morphological appearance similar to that of the vascular endothelium in vivo. Similarly, their apical cell surface is, as in vivo, nonthrombogenic. In contrast, when the cultures are maintained in the absence of FGF, the cells undergo within two to three passages structural and functional alterations that are incompatible with their in vivo morphological appearance and physiological function. Cultures maintained in the absence of FGF no longer adopt, upon reaching confluence, the configuration of a monolayer composed of small, closely apposed and nonoverlapping, cuboidal cells. Instead, confluent cultures deprived of FGF consist of large, overlapping cells which have lost the polarity of cell surfaces characteristic of the vascular endothelium. The apical cell surface becomes thrombogenic, as reflected by its ability to bind platelets, whereas fibronectin, which at confluence is normally associated only with the basal cell surface, can be found both on top of and underneath the cell layer. Among other changes, both sparse and confluent cultures maintained in the absence of FGF showed a greatly increased production of fibronectin. CSP-60, a cell surface protein whose appearance is correlative with the adoption of a cell monolayer configuration, can no longer be detected in cultures maintained in the absence of FGF. Overlapping endothelial cells maintained in the absence of FGF can also no longer function as a protective barrier against the uptake of ligands such as low density lipoprotein. Exposure of the culture to FGF induces a restoration of the normal endothelial characteristics concomitant with the adoption of a flattened cell monolayer morphology. These results demonstrate that, in addition to being a mitogen, FGF is involved in controlling the differentiation and phenotypic expression of the vascular endothelium. This is reflected by its effect on the morphological appearance, polarity of cell surfaces, platelet binding capacity, and barrier function of the vascular endothelium.
The use of fibroblast growth factor (FGF) has allowed the establishment of clonal endothelial cell populations derived from vascular territories as different in origin and age as the adult and fetal aortic arches, the fetal heart, and the umbilical vein (9, 14, 15). Low density cultures maintained in the presence of FGF proliferate actively and upon reaching confluence exhibit both the morphology and function characteristic of the vascular endothelium in vivo. These include: (a) formation of a confluent cell monolayer composed of highly flattened and closely apposed cells which no longer proliferate; (b) secretion of a fibrillar basement membrane composed mostly of fibronectin, proteoglycans, and collagen type IV; (c) a nonthrombogenic apical cell surface; (d) an active production of prostacyclins (PGI2); and (e) formation of an efficient barrier against a receptor-mediated uptake of low density lipoprotein (LDL) (1, 6, 9, 14, 15). In addition, it was observed that the formation of a cell monolayer composed of highly flattened and closely apposed cells which no longer proliferate; (b) secretion of a fibrillar basement membrane composed mostly of fibronectin, proteoglycans, and collagen type IV; (c) a nonthrombogenic apical cell surface; (d) an active production of prostacyclins (PGI2); and (e) formation of an efficient barrier against a receptor-mediated uptake of low density lipoprotein (LDL) (1, 6, 9, 14, 15, 27). In addition, it was observed that the formation of a high confluent cell monolayer is associated with (a) an appearance on the cell surface of a 60K molecular weight component (CSP-60) not present in actively growing cells or in a disorganized cell monolayer (29); (b) a disappearance of fibronectin from the apical cell surface and (1, 9, 14, 15); (c) a restriction of the lateral mobility of various cell surface receptor sites (28). In the present study, vascular endothelial cells were cultured in the absence of FGF to study whether the adoption of differentiated properties at confluence is dependent upon the presence of FGF during the phase of active cell growth. The results of these experiments have demonstrated that FGF, in addition to its potent mitogenic effect on endothelial cells, is involved in the adoption at confluence of a cell surface polarity and differentiated properties that are exhibited by the vascular endothelium in vivo.

MATERIALS AND METHODS

Materials

FGF was purified, as previously described, from bovine pituitary glands (7) and bovine brains (8). Both pituitary and brain FGF yielded single bands on polyacrylamide disk gel electrophoresis at pH 4.5. No bands were observed at pH 8.5. FGF was solubilized in medium containing 0.5% crystalline bovine serum albumin (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.).

Dulbecco's modified Eagle's tissue culture medium (DMEM, H-16) was obtained from Grand Island Biological Co., (GIBCO) Grand Island, N. Y., and calf serum from Irvine Scientific. Tissue culture dishes were obtained from Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif. Fungizone and Gentamycin from Gibco, and Gentamycin from Schering. [35S]Methionine, and [3H]serotonin were obtained from Amersham Corp., Arlington Heights, Ill., lactoperoxidase from Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif., and glucose oxidase from Sigma Chemical Co., St. Louis, Mo.

Cell Culture

Clonal populations of bovine vascular endothelial cells derived from the adult and fetal aortic arches and from the fetal heart were obtained as previously described (12, 13). Cells were routinely cultured in DMEM (H-16) supplemented with 10% bovine calf serum and maintained at 37°C in 10% CO2-humidified incubators. A fibronectin-free serum, rather than regular calf serum, was used in those experiments designed to detect the fibronectin associated with the cell surface. For this purpose, the fibronectin present in serum was first removed by subjecting the serum to affinity chromatography on a gelatin-Sepharose column as described (5). No fibronectin was detected in this serum by immunodiffusion in agarose against a specific rabbit anti-bovine plasma fibronectin (11). Cell stocks were passaged weekly at a split ratio of 1:64, and FGF (100 ng/ml) was added every other day. Confluent cultures were kept without a further addition of FGF. Similar results were obtained with confluent cultures that were continuously maintained with FGF (added every other day). The presence of Factor VIII antigen and the formation at confluence of a highly organized monolayer composed of flattened and closely apposed cells have been constant features of all subcultures of the vascular endothelial cells. Endothelial cells that were maintained without FGF were passaged every 7-10 d at a split ratio of 1:6 in the absence of FGF and could not be passaged beyond 30 generations because of precocious senescence. These cells lost, within two passages, their unique morphological organization at confluence, became considerably larger, and grew in a multiple layer. Cultures were used when sparse, subconfluent, or 7-10 d after reaching confluence. Cell density at confluence was 1.1 x 10⁶ cells/35-mm dish (260 pg protein/10⁶ cells) and 0.385 x 10⁶ cells/35-mm dish (1.462 μg protein/10⁶ cells) for cultures maintained with or without FGF, respectively. For growth-rate determinations, cells were seeded at an initial density of 1 x 10⁴ cells/35-mm dish in DMEM (H-16) containing 10% calf serum. The cultures were then maintained in the absence or presence of FGF (100 ng/ml, added every other day) and the medium was
replaced every 4 d. Triplicate plates were trypsinized every other day and the cell number was determined with a Coulter counter.

**Indirect Immunofluorescence Staining**

Cells maintained with or without FGF were grown on 12-mm glass coverslips in DMEM supplemented with 10% calf serum first depleted of its fibronectin content. At various times in culture, the coverslips were washed with DMEM containing 0.5% bovine serum albumin, fixed for 10 min with 3.7% formaldehyde in phosphate-buffered saline (PBS), and washed extensively with PBS. They were then incubated for 30 min with a 1:40 dilution of fluorescein isothiocyanate (FITC) conjugated rabbit anti-fibronectin antiserum, generously provided by Dr. C. R. Birdwell (Scripps Research Clinic, La Jolla, Calif.). This antiserum gave by immuno-electrophoresis one precipitin line against whole bovine plasma or serum and cross-reacted with fibronectin from several species (11). After three 15-min washes in PBS, the coverslips were incubated for 30 min at room temperature with a 1:20 dilution of fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit IgG (Meloy Co., Va.) in the same medium. Cultures were rinsed six times in PBS and once in distilled water and mounted for microscopy in buffered glycerol. Fluorescence was observed with a Leitz Orthoplan microscope under epi-illumination and photographed on Kodak Tri-X film with a Leitz Orthomat automatic exposure camera. In all cases where positive fluorescence was observed, the specificity of the staining was determined by control coverslips in which nonimmune rabbit serum was used in place of the anti-bovine plasma fibronectin antiserum. Under these conditions, little or no fluorescence could be observed. Cultures were also extracted with 0.5% Triton X-100 in PBS (5 min at room temperature with gentle shaking) to remove the cell monolayer and expose the extracellular matrix remaining on the coverslip. The coverslips were then subjected to indirect immunofluorescence as described above.

**Metabolic Labeling and Immunoprecipitation of Fibronectin**

Sparse and confluent cultures maintained with or without FGF were labeled with [35S]methionine (30 min, 450 μCi/ml), and the newly synthesized proteins were analyzed by two-dimensional gel electrophoresis (isoelectric focusing followed by SDS slab gel electrophoresis) as described (24).

**Iodination of Cell Surface Proteins**

Lectoperoxidase-glucose oxidase-catalyzed radioiodination of the cell monolayers was carried out in PBS in the presence of 111Na, lactoperoxidase, and glucose oxidase as described by Teng and Chen (25). Iodinated cells were washed five times with Ca2+−Mg2+−free PBS and lyed in buffer containing 15% glycerol, 2% SDS, 75 mM Tris-HCl (pH 6.8), and 2 mM PMSF and 2 mM EDTA to inhibit proteolysis. One mM N-ethylmaleimide and 1 mM iodoacetic acid were added to block free sulphydryl groups (18). The cell lysates were then boiled for 2 min to denature nucleic acids and proteins. To reduce disulfide bonds, DTT was added to a concentration of 0.1 M before the boiling step (18).

**Polyacrylamide Gel Electrophoresis**

Samples containing 10,000–50,000 protein bound cpm were applied to exponential gradient (5–16%) polyacrylamide slab gels with a 3% stacking gel as described (20). Two-dimensional electrophoresis was performed as described (24) (nonequilibrium, pH gradient 3.5–10 electrophoresis followed by SDS gel electrophoresis on a 10–16% gradient polyacrylamide slab gel). After electrophoresis, the slab gel was fixed in 7% acetic acid, dried, and subjected to autoradiography on Kodak NS-2T X-ray film for 8–72 h.

**Binding of Platelets to Cultured Endothelial Cells**

Platelets were prepared from 60–120 ml of human whole blood according to the procedure described by Tollefsen et al. (26). To observe platelet binding, 2 × 106 platelets were added to confluent endothelial cell cultures in 35-mm dishes containing 1 ml of DMEM supplemented with 0.25% bovine serum albumin. After 30-min incubation at 37°C in a humidified CO2 incubator, the cultures were washed ten times with the same medium and viewed by phase contrast microscopy. For quantification of platelet binding and serotonin release from platelet granules, the platelets were pre-incubated for 30 min with 3H-serotonin (0.4 μCi/ml). After incubation of the labeled platelets with the cells, the culture medium was aspirated and the bound serotonin was measured.
was removed and the platelets were collected by centrifugation. The platelets were then solubilized in 0.2 M NaOH, as were the cells with bound platelets remaining on the culture dish. The three fractions (platelets, cells, and platelet-free supernate) were then counted separately in Bray's solution with a Beckman LS 8000 Scintillation counter (Beckman Instruments, Inc., Palo Alto, Calif.).

Karyotyping

Endothelial cultures maintained with and without FGF were karyotyped as described by Hsu (17). 30-50 Giemsa-band metaphase cells were counted and photographed with a Nikon Biophot equipped with an automatic camera and ×100 oil immersion objective.

RESULTS

Morphological Appearance and Growth Properties

Vascular endothelial cells maintained in the presence of FGF divide rapidly (doubling time = 18 h) when seeded at either a high (up to 1:1,000) or low split ratio (Figs. 1 and 2). Upon reaching confluence, the cells adopt a morphological configuration similar to that of the confluent culture from which they originated. The cells are closely apposed, cuboidal in shape, and form a highly flattened cell monolayer in which no cell overlapping could be observed (Fig. 3 D). A similar growth-rate and a similar morphological appearance at confluence were observed even when the cells were seeded at a clonal density (1–10 cells/cm²) provided FGF was added every other day to the culture. In contrast, seeding of the same cells in the absence of FGF resulted, even at a low (1:6) split ratio, in a much longer doubling time (60–78 h) (Figs. 1 and 2) and in a strikingly different morphology (Fig. 3 A and B). When seeded at a high split ratio (1:128) and in the absence of FGF, the cells proliferated poorly and became highly vacuolated and senescent (Fig. 2). The alterations
in growth behavior and morphological appearance were best demonstrated after 3-4 passages (15-20 generations) in the absence of FGF. The cells, by then four- to six-fold larger in size, failed to adopt a nonoverlapping monolayer configuration even after being split at a 1:4 ratio. Instead, at sparse density they were flattened, enlarged, and spread out (Fig. 3 A), and at confluence they grew on top of each other, leaving intercellular spaces (Fig. 3 B). These cells exhibited a short lifespan, as reflected by vacuolization and cell degeneration after 30 generations in the absence of FGF.

**Localization and Synthesis of Fibronectin**

**Immunofluorescence studies:** The distribution pattern of fibronectin in endothelial cultures maintained with or without FGF was studied by indirect immunofluorescence using a specific rabbit anti-bovine plasma fibronectin antiserum and fluorescein-conjugated goat anti-rabbit gamma globulin. To avoid detection of the fibronectin present in the tissue culture medium, bovine serum was subjected to affinity chromatography on a gelatin-Sepharose column to remove the fibronectin before being added to the tissue culture medium. In the presence of FGF, marked changes in the surface distribution of fibronectin were observed as cells that were not yet in contact proceeded to form an unorganized subconfluent culture and, later, a tightly packed monolayer of cells. Single cells not yet in contact did not stain for fibronectin (Fig. 4 B). In sparse cultures, fibronec-
tin was found on the apical cell surfaces, but, as the cell density increased, it was preferably concentrated in the areas of contacts between cells (Fig. 4 D), thus reflecting a possible involvement in cell-cell interactions. Fibronectin was slightly or no longer detectable late at confluence (Fig. 4 F), thus confirming previous results (9, 14, 15) on the acquisition of a cell surface polarity associated with the formation of a confluent vascular (1) or corneal (11) endothelial cell monolayer. In contrast, cells that were cultured in the absence of FGF showed a fibrillar distribution of fibronectin over the entire apical cell surface, both at low cell density before the formation of cell-cell contacts (Fig. 4 A) and late at confluence (Fig. 4 E).

Fibronectin was shown to be a major component of the fibrillar matrix that underlies a confluent endothelial cell monolayer (1, 19, 22). Immunofluorescence staining of the extracellular material left after removing the confluent cell layer with Triton X-100 revealed a massive accumulation of fibronectin either with (Fig. 4 H) or without (Fig. 4 G) a prior addition of FGF. Endothelial cells that were cultured in the absence of FGF therefore resembled fibroblasts (16) or smooth muscle cells (3) in having fibronectin associated with both their upper and lower cell surfaces. The expression of fibronectin only underneath the confluent cell layer might therefore be considered to be a unique property of endothelial cells that are maintained in the presence of FGF and that adopt at confluence the characteristic morphological appearance of the vascular endothelium in vivo, which is that of a monolayer composed of closely apposed and nonoverlapping cells.

**Metabolic Labeling:** Sparse and confluent endothelial cultures maintained with or without FGF were exposed (20 h) to [35S]methionine, and both the cell layer and tissue culture medium were analyzed for the presence of 35S-labeled fibronectin before and after immunoprecipitation with antifibronectin antiserum (Figs. 5 and 6, Table I). The higher production of fibronectin by cells that were maintained in the absence of FGF was best demonstrated by subjecting the tissue culture medium collected from either sparse or confluent cultures to immunoprecipitation with antifibronectin. These cells showed, on the basis of cell protein, little or no difference in the total amount of 35S-labeled TCA-precipitable material secreted into the medium. When calculated per cell, this value was higher in the absence than in the presence of FGF for both sparse (three-fold) and confluent (eight-fold) cultures (Table I). As shown in Table I, antifibronectin precipitated 1.7-3.2% of the total TCA-precipitable 35S-labeled proteins secreted into the medium of cells that were cultured with FGF, as compared to 18-25% of the total 35S-labeled proteins precipitated from the culture medium of cells that were maintained in the absence of FGF. When analyzed on SDS polyacrylamide gels (Fig. 5 A and F), more than 90% of the immunoprecipitated radioactivity comigrated with fibronectin. On the basis of the immunoprecipitation values, it can be calculated that sparse and confluent cultures maintained in the absence of FGF secreted into the medium 30- and 50-fold more fibronectin per cell than sparse and confluent cells cultured in the presence of FGF, respectively (Table I). The increased production of fibronectin by both sparse and confluent endothelial cells maintained in the absence (Fig. 5 D and E, I and J), rather than in the presence (Fig. 5 B and C, G and H) of FGF, was directly demonstrated by subjecting aliquots of the 35S-labeled medium to SDS-PAGE. Fibronectin was found to be the major 35S-labeled protein present in the medium. Under nonreduced conditions (Fig. 5 A–E), it existed predominantly as dimers (460K), although higher disulfide-bonded complexes were also present. The fibronectin secreted into the medium was further identified by an immunoprecipitation with antifibronectin (Fig. 5 A and F).

A higher production of fibronectin by sparse and confluent cultures maintained in the absence rather than in the presence of FGF was also observed when cell extracts, rather than the tissue culture medium, were analyzed for the presence of 35S-labeled fibronectin (Fig. 5 K–N). The cellular, newly synthesized fibronectin was further identified by double immunoprecipitation with antifibronectin antiserum (Fig. 6) and was, in fact, hardly or not detected either before or after precipitation, in a total extract of cells that were maintained with FGF (Fig. 6 A and B, E and F). Because insoluble material was first removed by centrifugation, only a partial recovery of the cell extract was obtained by extraction in the immunoprecipitation medium (0.5% Triton X-100, 0.1% SDS) (Fig. 6) as compared with a solubilization in 2% SDS (Fig. 5, lanes K–N).

To detect differences other than in fibronectin production and in particular in the range of lower molecular weight proteins, sparse and confluent cultures maintained with and without FGF were pulsed with [35S]methionine (400 µCi/ml, 30 min), and the newly synthesized proteins were analyzed by a two-dimensional gel electrophoresis (isoelect-
tric focusing in the first dimension and SDS polyacrylamide gradient (10–16%) gel electrophoresis in the second dimension. The most prominent differences thus detected are marked in Fig. 7. A number of differences in labeling intensities of various proteins can be noted between cultures maintained with (Fig. 7 A and C) or without (Fig. 7 B and D) FGF. Some proteins were observed to be made to a greater extent in the absence than in the presence of FGF, and vice-versa. The relation of these differences to the mitogenic effect of FGF and to changes in growth behavior and mode of interaction among cells is currently being studied. It can be seen, however, that the overall protein pattern is similar under all conditions, except when a highly organized endothelial cell monolayer is formed. This suggests that the major differences in labeling represent a difference between growing and resting cultures rather than changes induced by the FGF alone. Although cells in a resting endothelial monolayer produced fewer proteins (in terms of types and total incorporation of $[^35]S$methionine) than growing cells, the amount of one protein in particular (double arrowhead) was considerably larger in resting (Fig. 7 C) than in growing cells maintained with (Fig. 7 A) or without (Fig. 7 B and D) FGF. Preliminary studies demonstrate that this protein migrates in a manner similar to the 58,000 mol wt intermediate (10-nm) filaments. Experiments which will allow a quantitative analysis and a more specific identification of this protein are now in progress. The wide occurrence of intermediate filaments in confluent endothelial cell cultures might reflect a role in maintaining the flattened and closely apposed morphology of the endothelial cells as well as in the restriction of surface receptor lateral mobility observed when the cells adopt a monolayer configuration (28).

**Cell Surface Proteins Detected by Radioiodination**

**FIBRONECTIN:** Sparse and confluent cultures of endothelial cells maintained with or without FGF were subjected to the lactoperoxidase-catalyzed iodination to study further the changes in quantity and surface localization of fibronectin, particularly in relation to other cell surface proteins. As demonstrated in Fig. 8, little or no fibronectin was detected in sparse cultures maintained in the presence of FGF (lanes A and G), whereas sparse cultures grown in its absence already showed, before the formation of cell-cell contacts (lanes D and J), more fibronectin than that found in confluent cultures grown in the presence of FGF (lanes B and H). In fact, in cultures maintained without FGF, fibronectin was the major component susceptible to iodination both when the cells were sparse and in cultures that were kept at confluence for prolonged periods of time (7–12 d) (lanes E and K). These results together with the immunofluorescence and $[^35]S$-labeling experiments indicate that the production and distribution of fibronectin are sensitive to changes in cell organization and growth behavior induced by FGF. The

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**Figure 4** Indirect immunofluorescence localization of fibronectin in endothelial cultures maintained with or without FGF. Vascular aortic endothelial cells maintained with or without (three passages) FGF were stained with rabbit anti-bovine plasma fibronectin and FITC goat anti-rabbit IgG as described in Materials and Methods. Fluorescent fields were photographed with a Leitz Orthomat automatic exposure camera. Exposure times ranged from 15 s to a maximum of 2 min. Exposure times greater than 2 min were stopped manually at 2.5 min (Fig. 4 B and F). All negatives were printed at the same contrast (A and B) Sparse cultures before the formation of cell-cell contacts. Fibronectin is detected on top of cells that are maintained in the absence (A) but not in the presence (B) of FGF. (C and D) Sparse and subconfluent cultures. Fibronectin is detected on top of cells that are maintained with (D) or without (C) FGF. This location is particularly evident in the absence of FGF, whereas in its presence the fibronectin tends to concentrate in the areas of cell-cell contact. (E and F) Confluent cultures. Fibronectin is slightly or not detectable on top of cells that were maintained with FGF and have adopted the configuration of a highly organized monolayer (F). In contrast, fibronectin is present in large quantities on top of cells that were cultured in the absence of FGF (E). (G and H) Extracellular matrix left after removing the confluent cell layer with Triton X-100 (0.5%, 5 min at 37°C). Fibronectin associated with the extracellular matrix is present in cultures maintained with (H) or without (G) FGF. Differences in organization can be observed—the fibronectin produced in the absence of FGF is deposited in thinner fibers and is distributed in various directions rather than in a concentric manner.
FIGURE 5  Electrophoresis of [35S]methionine-labeled proteins present in cell extracts and tissue culture medium of sparse and confluent endothelial cultures maintained with or without FGF. Sparse (actively growing) and confluent (10 d after reaching confluence) endothelial cultures maintained with or without (three passages) FGF were exposed to [35S]methionine (65 μCi/ml, 20 h) in DMEM containing 10 μM methionine and 0.5% bovine serum. The medium was collected and 0.1-ml aliquots were diluted with an equal volume of 2× sample buffer. 10- to 30-μl aliquots containing 18,695, 28,695, 45,000, and 49,835 protein-bound (TCA-precipitable) cpm were applied to lanes (B and G), (C and H), (D and I), and (E and J), respectively. The cell layer was washed six times, solubilized in sample buffer, and 50,000 TCA-precipitable cpm were applied to each lane (lanes K–N). Samples were run before (lanes A–E) and after (lanes F–N) reduction with 0.1 M DTT on a gradient (5–16%) polyacrylamide slab gel. (A and F) Immunoprecipitation pattern (antifibronectin followed by anti-IgG) of growth medium taken from sparse endothelial cells cultured in the absence of FGF. (B and G) Proteins secreted into the medium by sparse cells maintained in the presence of FGF. (C and H) Proteins secreted by confluent cells cultured in the presence of FGF. (D and I) [35S]-labeled proteins secreted into the medium by sparse cells that were maintained in the absence of FGF. (E and J) Proteins secreted into the medium by sparse cells maintained in the presence of FGF. (K and L) Cell extracts of sparse (K and M) and confluent (L and N) endothelial cultures maintained with (K and L) or without (M and N) FGF. Arrows mark the positions of fibronectin dimers (460K, nonreduced samples) and monomers (230K, reduced samples).

Radioiodination experiments also demonstrated that fibronectin in cells maintained with or without FGF is disulfide- or otherwise (transglutaminase-) bonded to form dimers, trimers, and higher complexes which hardly entered the running gel. Most of these aggregates were precipitated by antifibronectin antiserum and gave rise to a 230K component (fibronectin monomer) after reducing the samples with DTT (Fig. 8 H, J, and K). The presence in nonreduced samples of residual amounts of monomeric fibronectin might have been due to a proteolytic cleavage of dimeric and polymeric fibronectin (2). Both the dimers and higher complexes of fibronectin were highly sensitive to proteolysis and were largely removed from the cell surface by pretreating the cultures with trypsin (0.2 μg/ml, 1 h, 37°C) (Fig. 8 C, F, I, and L), thus confirming the results of others (18, 25, 30) regarding the trypsin sensitivity of fibronectin.

CSP-60: In a previous study (29), we identified a 60K mol wt cell surface protein (CSP-60) exposed to iodination by lactoperoxidase only in endothelial cells cultured in the absence of FGF. (K–N) Cell extracts of sparse (K and M) and confluent (L and N) endothelial cultures maintained with (K and L) or without (M and N) FGF.
FIGURE 6  Metabolic labeling pattern $[^{35}S]$methionine of sparse and confluent endothelial cultures maintained with or without FGF. Sparse and confluent endothelial cultures maintained with or without FGF were exposed to $[^{35}S]$methionine as described in the legend to Fig. 5. The cell layer was washed, extracted in immunoprecipitation medium, and samples were subjected to double immunoprecipitation with either antifibronectin (lanes E–H) or nonimmune rabbit serum (lanes I–L) as described in Materials and Methods. The immunoprecipitates as well as the original samples (lanes A–D) were analyzed on gradient (5–15%) polyacrylamide gels after reduction with 0.1 M DTT. (A, E, and I) Sparse cultures maintained with FGF. (B, F, and J) Confluent cells cultured in the presence of FGF. (C, G, and K) Sparse cultures maintained without FGF. (D, H, and L) Confluent cells that were not exposed to FGF. $^{35}$S-labeled fibronectin is detected only or mostly in the absence of FGF and after immunoprecipitation with antifibronectin (lanes G and H).

regardless of their origins and ages. It was not detected either in actively growing or in unorganized endothelial cell cultures. Likewise, cell types such as vascular smooth muscle cells that grow in multiple layers did not contain CSP-60. The appearance of CSP-60 on the surface of either growing or resting endothelial cells maintained in the presence or absence of FGF was studied by subjecting the cultures to the lactoperoxidase-catalyzed iodination. As demonstrated in Fig. 8, CSP-60 was not detected in sparse cultures maintained with (lanes A and G) or without (lanes D and J) FGF. In contrast, it was present as a major cell-surface component in confluent cultures that were maintained in the presence of FGF and had adopted a cell monolayer configuration (Fig. 8 B and H). The same iodination pattern was obtained whether or not FGF was added repeatedly after the cells had adopted this configuration. In contrast to these results, little or no CSP-60 was detected, even late at confluence, in endothelial cells that were cultured in the absence of FGF and therefore grew on top of each other (Fig. 8 E and K). In some experiments, these cultures showed very small residual amounts of CSP-60, which could have been present in areas still showing a monolayer-like configuration. As demonstrated by the electrophoresis pattern before and after reducing the samples with DTT and by a two-dimensional SDS gel electrophoresis (29), CSP-60 exists...
Karyotype

Previous results (9, 14, 15) had demonstrated that, provided FGF is included in the tissue culture medium, no detectable changes in the normal karyotype and no loss of differentiated properties could be observed in endothelial cells maintained in culture for as long as 200 cell generations. As demonstrated in Fig. 9, vascular endothelial cells that were cultured (seven passages) in the absence of FGF still exhibited a normal karyotype, indicating that the observed changes in morphological appearance, growth behavior, and cell surface properties are not due to chromosomal aberrations insofar as chromosome number and shape are concerned.

**Interaction with Platelets**

Sparse and confluent endothelial cells maintained with FGF had a nonthrombogenic apical surface to which platelets cannot adhere. Thus, no platelets or <1 platelet/cell were found on top of cells that were incubated (30 min, 37°C) with human platelets (2 × 10⁸ platelets/ml) and washed extensively (Fig. 11 F). In contrast, endothelial cells maintained without FGF exhibited under the same conditions a high platelet-binding capacity (20–50 platelets/cell) both at a sparse and confluent (Fig. 11 C) cell density. As shown in Fig. 11 C, the platelets were bound singly to the apical surface of these cells and no aggregation of the attached platelets took place. To determine whether this interaction is associated with a release reaction, confluent cultures maintained with or without FGF were incubated with platelets that were prelabeled with ³H-serotonin. The reaction medium containing unattached platelets and any serotonin that might have been released during binding was then removed, the platelets were collected by centrifugation, and the cell layer was extensively washed and solubilized in 0.1 N

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**Table I**

| Cells          | Total ³S-labeled proteins* cpm × 10⁻⁶/10⁶ cells | ³S-labeled material precipitated with antifibronectin cpm × 10⁻⁶/10⁶ cells | % Fibronectin in total ³S-labeled proteins |
|---------------|-----------------------------------------------|---------------------------------------------------------------------------|------------------------------------------|
| (+) FGF cultures | Sparse 11.6 | 0.195 | 1.7 |
|               | Confluent 3.8 | 0.120 | 3.2 |
| (-) FGF cultures | Sparse 26.9 | 6.45 | 24.4 |
|               | Confluent 32.6 | 5.76 | 17.7 |

Sparse and confluent endothelial cultures were maintained with or without FGF as described in the legend to Fig. 5 and exposed to ³⁵S]methionine (65 μCi/ml, 20 h) in DMEM containing 10 μM methionine and 0.5% bovine serum as described in the legend to Fig. 5. 10-μl aliquots of the growth medium were taken for precipitation with 10% boiling TCA, and the entire 1 ml of medium was subjected to double immunoprecipitation with 10 μl of rabbit anti-bovine fibronectin antiserum followed by 10 μl of goat anti-rabbit IgG antiserum as described under Materials and Methods. The same amounts of fibronectin were precipitated by using either 5 μl or 50 μl of each of these antisera preparations.

* Sparse and confluent endothelial cells maintained in the presence of FGF contained 320 and 260 μg protein per 10⁶ cells, whereas sparse and confluent cells maintained in the absence of FGF contained 1,810 and 1,462 μg protein per 10⁶ cells, respectively.

† Nonspecific precipitation with nonimmune rabbit serum was carried out under each condition, and the values were subtracted from those obtained with antifibronectin. Nonspecific precipitation did not exceed 1% of the total ³⁵S-labeled proteins secreted into the medium.
FIGURE 7  Two-dimensional electrophoresis pattern of proteins synthesized by sparse and confluent vascular endothelial cells maintained with or without FGF. Endothelial cultures maintained with or without FGF were exposed to [35S]methionine (450 μCi/ml, 30 min, 37°C), washed, and lysed with isoelectric focusing sample buffer (9.5 M urea, 2% NP-40, 20% ampholytes, pH 3.5-10, 5% β-mercaptoethanol). 500,000 protein-bound cpm were then subjected to two-dimensional gel electrophoresis according to the procedure of O'Farrel et al. (24). (A) Sparse, actively growing endothelial cells seeded in the presence of FGF. (B) Sparse endothelial cultures maintained (four passages) without FGF. (C) Confluent, highly organized and resting monolayer of endothelial cells that were cultured (22 passages) in the presence of FGF. (D) Confluent, overlapping and slowly dividing endothelial cells maintained (four passages) without FGF. Arrows mark the main differences between the four autoradiograms (exposure time was 3 d).

NaOH. The cells, platelets, and platelet-free supernate were counted separately to determine the distribution of [3H]serotonin. As shown in Table II, the interaction between platelets and endothelial cells maintained with or without FGF was not associated with any serotonin release beyond that spontaneously released during incubation (30 min, 37°C) of platelets in the absence of cells. The lack of serotonin release was not due to defects in the release reaction, since exposure to thrombin (1 IU/ml) induced the platelets to secrete 80-90% of their [3H]serotonin content (Table II). When the
Figure 8  SDS-PAGE of lactoperoxidase iodinated sparse and confluent endothelial cultures maintained with or without FGF. Endothelial cultures (maintained with or without FGF in a medium containing a fibronectin-depleted serum), were iodinated before or after exposure to trypsin with ¹¹¹I-iodine and lactoperoxidase, lysed, and analyzed by a gradient (5–15%) polyacrylamide slab gel electrophoresis either before (lanes A–F) or after (lanes G–L) reduction with 0.1 M DTT. (A and G) Sparse cultures maintained in the presence of FGF—almost no contacts among the cells. Fibronectin and CSP-60 are slightly or not detected. (B and H) A confluent monolayer of cells cultured in the presence of FGF. Both CSP-60 and fibronectin become susceptible to iodination; CSP-60 appears as a major band. (C and I) Confluent cultures exposed to a mild trypsinization (0.2 μg/ml, 45 min, 37°C) and then iodinated. Fibronectin is largely removed, whereas CSP-60 is affected little or not at all. (D and J) Sparse cultures before the formation of cell-cell contacts and maintained in the absence of FGF. Fibronectin is highly susceptible to iodination. (E and K) Confluent endothelial cultures maintained in the absence of FGF. Fibronectin appears as the major component, whereas CSP-60 is slightly or not detectable. (F and L) Confluent cultures iodinated after a mild trypsinization. Fibronectin is largely removed.

Radioactivity associated with the cell layer was measured, it was found, as already observed (Fig. 11 C and F), that endothelial cells maintained without FGF bind platelets to an extent that is 9- and 30-fold higher, when calculated per plate and per cell, respectively, than cells that were cultured in the presence of FGF. Trypsinization (0.5 μg/ml, 2 h, 37°C), which caused no release or rounding up of cells but removed the fibronectin from the upper surface of cells that were cultured in the absence of FGF (as detected by the lactoperoxidase-catalyzed iodination) (Fig. 8 F and L), decreased the adherence of platelets by no more than 40%.

Readdition of FGF to Endothelial Cells Maintained in its Absence

Endothelial cells maintained for three passages in the absence of FGF were exposed to FGF to study whether or not and under what conditions the cells can revert in terms of growth behavior, morphological appearance, and cell surface properties. As demonstrated in Fig. 10, cells that were seeded at a low density (split ratio 1:16) showed a normal proliferative response to readdition of FGF, as indicated by a four-fold decrease in doubling time (18 h) and increase in cell density at confluence (900 cells/mm²). In terms of morpho-
FIGURE 9 Karyotype of bovine vascular endothelial cells cultured in the presence and absence of FGF. (A) Bovine aortic endothelial cells maintained continuously (160 generations) in the presence of FGF. (B) The same cells as in A after seven passages (28 generations in the absence of FGF). In both cases, 30-50 Giemsa-band metaphase cells were counted and consisted of 58 normal acrocentric or telocentric chromosomes, one normal, large, submetacentric X chromosome, and one small, submetacentric Y chromosome. × 100.

For study of whether an active cell proliferation is required to induce a reversal by FGF, cells were reexposed to FGF when subconfluent or late at confluence. It was found that as the cell density and degree of overlapping increased, the cells became less able to regain their normal differentiated properties. Thus, endothelial cultures that formed at confluence a multiple cell layer showed a very limited degree of reversion, as measured by changes in morphological appearance, surface iodination pattern, and platelet binding capacity. In contrast, readdition of FGF at a sparse or subconfluent density led, within 5–7 d, to the adoption by most of the cultures of both a monolayer configuration (Fig. 11 E) and a nonthrombogenic apical surface (not shown), and this was associated with only 1–2 divisions of the entire cell population. This reversion was, however, incomplete, in that 10–20% of the culture area showed an unorganized morphology even after being maintained for 10 d at confluence (Fig. 11 E). When tested for surface iodination pattern (Fig. 12 C and H), CSP-60 reappeared in the reverted culture as a major cell-surface protein, whereas fibronectin was detected in reduced but still large amounts, when compared to confluent cultures continuously maintained with FGF. Indirect immunofluorescence had also demonstrated that a considerable proportion of the fibronectin remained on the apical cell surface (Fig. 11 H), despite the addition of FGF and the associated decrease in fibronectin synthesis and secretion. These results also indicate that endothelial cells can be induced to adopt a monolayer configuration and nonthrombogenic properties despite the presence (albeit in reduced amounts) of fibronectin on their apical cell surface.

DISCUSSION

FGF is a potent mitogen for vascular endothelial cells and is an essential factor in developing clonal
TABLE II
Binding of Platelets to Vascular Endothelial Cells
Maintained with or without FGF

| Cells                  | Cell-bound platelets (14C-serotonin) | Released 14C-serotonin |
|------------------------|-------------------------------------|------------------------|
|                        | cpm/dish                            | cpm/10^6 cells         |
| +FGF cultures          | 1,192                               | 1,748                  |
| -FGF cultures          | 9,504                               | 50,553                 |
| -FGF cultures pre-     | 5,227                               | 27,803                 |
| treated with trypsin*  |                                     |                        |
| No cells               |                                     |                        |
| No cells + thrombin (1 U/ml) |                                     |                        |

2 × 10^8 platelets prelabeled with 14C-serotonin were incubated (30 min at 37°C in DMEM containing 0.25% BSA) with confluent endothelial cultures that were maintained with (during the active phase of growth) or without (three passages) FGF. Platelet binding and serotonin release were determined as described in Materials and Methods. The amount of serotonin release was in all cases equivalent to that spontaneously released by platelets during a 30-min incubation without cells. Treatment of 2 × 10^8 platelets with thrombin (1 U/ml) induced a 90% release of the 14C-serotonin that was introduced into the platelets.

* Worthington X2 crystallized trypsin (0.5 µg/ml, 2 h 37°C in DMEM).

The present results have demonstrated that cells maintained in the absence of FGF for more than three passages exhibit, in addition to a much slower growth rate, morphological as well as structural and functional alterations that are incompatible with their appearance and behavior in vivo. These include: (a) a failure to adopt at confluence the configuration of a cell monolayer composed of flattened, closely apposed, and nonoverlapping cells; (b) the loss of a cell surface polarity, as demonstrated by the presence late at confluence of fibronectin associated with both the apical and basal cell surfaces, and by thrombogenicity (platelet binding capacity) of the apical cell surfaces; (c) an increased production and secretion of fibronectin populations of endothelial cells that exhibit most if not all of the characteristics of the vascular endothelium in vivo (9, 14, 15). The experimental approach taken in the present study was to compare endothelial cells maintained with and without FGF to study whether changes other than those related to cell proliferation can be induced by the presence of FGF. Of particular interest in this regard is the adoption at confluence of a highly flattened, nonoverlapping and closely apposed cell organization as well as of a cell surface polarity. This polarity reflects an advanced stage of differentiation in which the upper cell surface is non-thrombogenic and no longer covered with fibronectin, whereas the basal cell surface is closely associated with a highly thrombogenic fibrillar matrix composed mostly of fibronectin and collagen. It is important to study how endothelial cells can gain and lose such characteristics to elucidate the factors that allow the endothelium of the large vessels to form a nonthrombogenic surface, to resist high pressure and shear forces, and to function as a selective barrier against plasma components and, in particular, lipoproteins.

The present results have demonstrated that cells maintained in the absence of FGF for more than three passages exhibit, in addition to a much slower growth rate, morphological as well as structural and functional alterations that are incompatible with their appearance and behavior in vivo. These include: (a) a failure to adopt at confluence the configuration of a cell monolayer composed of flattened, closely apposed, and nonoverlapping cells; (b) the loss of a cell surface polarity, as demonstrated by the presence late at confluence of fibronectin associated with both the apical and basal cell surfaces, and by thrombogenicity (platelet binding capacity) of the apical cell surfaces; (c) an increased production and secretion of fibronectin...
Morphological appearance, fibronectin distribution, and platelet binding capacity of overlapping endothelial cells before and after reexposing the cultures to FGF. Endothelial cells maintained for three passages in the absence of FGF were seeded at a split ratio of 1:8. Control cultures (not exposed to FGF) and reorganized cultures (exposed to FGF on day 2 or day 7 after seeding and every other day thereafter) were tested for morphological appearance (A–F), platelet binding capacity (C and F), and fibronectin distribution (G–I) as described under Materials and Methods. (A) Sparse cells, 3 d after seeding, with no addition of FGF. (B and G) Confluent culture of overlapping cells maintained (14 d) without FGF. The cells grow on top of each other and in various directions (B) and are covered by an extensive meshwork of fibronectin (G). (C) Confluent (−FGF) endothelial culture incubated (30 min, 37°C) with human platelets (2 × 10⁸ platelets/ml) and washed extensively. The cell surface is covered with platelets that are attached singly and do not form aggregates. (D) Sparse cells as in A, 3 d after exposure to FGF (6 d in culture). The cells start to divide faster, become smaller and less spread. (E and H) The same endothelial cells as in A exposed to FGF (added every other day) at a subconfluent density (8 d after seeding) and observed 8 d later. Most of the culture adopts the configuration of a cell monolayer composed of small, closely apposed and nonoverlapping cells (E). The amounts of fibronectin detected on the apical cell surfaces are largely reduced (H). Some areas (20–30% of the entire culture) fail to organize and show both cell overlapping (E) and the presence of fibronectin on top of the cells (H). (F and I) The same cells as in A exposed to FGF starting on day 3 after seeding and tested for platelet binding capacity (F) and fibronectin distribution (I) 2 wk later, when the entire culture adopted the cell monolayer configuration characteristic of vascular endothelial cells that are continuously maintained with FGF. (F) Very few or no platelets can be seen to be attached on top of the cells, as in confluent endothelial cultures that were not subjected to a withdrawal of FGF. (I) Very small amounts or no fibronectin can be detected on top of the cells.

tin by both sparse and confluent cells; and (d) a lack of CSP-60 (detected by the lactoperoxidase-catalyzed iodination technique), a cell surface protein susceptible to iodination only in endothelial cells that adopt a monolayer configuration. In addition, as previously shown (6, 27), endothelial cells maintained in the absence of FGF failed to form an effective barrier against low density lipoprotein, which is the primary cholesterol carrier present in blood. The physiological significance of
FIGURE 12 Cell-surface iodination pattern of overlapping and reorganized endothelial cells before and after being reexposed to FGF. Endothelial cells maintained for three passages in the absence of FGF were seeded at a split ratio of 1:8, and FGF was added (every other day) starting on day 3 or day 7 after seeding. Control cultures (not exposed to FGF) and reorganized cultures (reexposed to FGF) were iodinated at confluence (14 d after seeding) and analyzed by gradient (5-15%) PAGE before (lanes A-E) and after (lanes F-J) reduction with 0.1 M DTT. (A and F) A confluent monolayer of endothelial cells that were continuously maintained in the presence of FGF. CSP-60 appears as a major band. (B and G) Confluent but unorganized endothelial culture maintained for four passages without FGF. Fibronectin appears as a major band; CSP-60 is missing. (C and H) Same cells as in B and G exposed to FGF at a subconfluent density (7 d after seeding) and labeled 7 d later. Most of the culture adopts a monolayer configuration, although some unorganized areas (~20% of the culture) are still present. Both fibronectin and CSP-60 are exposed for iodination. (D and I) The same cells as in B and G exposed to FGF at a subconfluent density (3 d after being split at a 1:8 ratio) and labeled 12 d afterwards, when the cells were highly organized and closely opposed. CSP-60 appears as a major band, whereas fibronectin is detected in small amounts as in cells that are maintained continuously with FGF. (E and J) (~FGF) culture exposed when subconfluent to a medium conditioned by a subconfluent monolayer of endothelial cells and iodinated 7 d later.

The alterations listed above is in particular demonstrated by the inability of non-contact-inhibited (minus FGF) endothelial cells to protect the subendothelial layer against overaccumulation of LDL cholesterol. This might initiate the accumulation of foam cells and lead later on to the formation of an atherosclerotic plaque. Furthermore, by having a thrombogenic surface exposed to the medium, these cells can serve as a nucleus for platelet adherence, blood clotting, and thrombus formation.

The notion of a factor simultaneously inducing apparently contradictory effects such as cell proliferation and differentiation might at first look surprising. Whether or not FGF is directly involved, via a mechanism distinct from its mitogenic activity, in the adoption of the monolayer configuration and cell surface polarity characteristic of endothelial cells has yet to be studied. A simple explanation might be that the presence of FGF is required for the formation of a cell monolayer, while the other differentiated attributes are controlled by secondary factors like the unique cell-cell adhesive interactions established at confluence. That this might not be the case is suggested by the finding that some of the FGF-dependent alterations (increased production of fibronectin, platelet binding capacity) were already observed at a sparse cell density and even before the formation of cell-cell contacts, suggesting, in turn, a direct relation to the actual withdrawal of FGF. On the other hand, the appearance of CSP-60 and the removal of fibronectin from the apical cell surface seem to depend more on a prior formation of the appropriate cell-cell contacts. The expression of fibronectin on the apical surfaces of 3T3 cells was also found to be controlled by epidermal growth factor (EGF) and in a manner dissociable from its mitogenic activity (4). With these cells, however, a stimulated production of fibronectin, rather than an inhibitory effect, was induced, thus leading to a differentiated state characteristic of a fibroblastic cell type. It should also be emphasized that each of the alterations observed in the absence of FGF represents a phenotypic rather than a permanent genetic modification, because the readdition of FGF was associated with a restoration of the normal growth rate, morphological appearance, and membrane properties. Endothelial cells maintained in the absence of FGF also showed no chromosomal aberrations insofar as chromosome number and morphology are concerned. Vascular endothelial cells maintained in the absence of FGF acquire a gross morphology as well as some cell-surface and metabolic properties similar to those of vascular smooth muscle cells. The reversal
of the observed changes by reexposing the cultures to FGF indicates, however, that the effects of withdrawing the FGF from cultured endothelial cells are not due to an overproliferation of a residual population of vascular smooth muscle cells which might have taken place in the absence of FGF.

Our results on the reversion induced by FGF indicate that this can be achieved under well-defined conditions and depends on the number of passages in the absence of FGF as well as on the cell density and organization at the time of reexposure to FGF. The reorganization of cells into a closely apposed monolayer of cells required at least one to two cell doublings and was always associated with an appearance of CSP-60, inhibition of fibronectin production, and secretion into the tissue culture medium, and the acquisition of nonthrombogenic properties. On the other hand, an actual removal of fibronectin already present on the apical cell surfaces was only partially achieved. The finding that only a limited reversion, if any, was obtained when unorganized cultures were reexposed to FGF late at confluence suggests that the spatial organization might, among the other factors, determine whether or not and to what extent cells will respond to FGF. A dependence on cell shape and spatial organization was already found to be a major factor in determining the sensitivity of corneal epithelial cells to EGF (10, 15). Preliminary results from this laboratory (Fig. 12 E and J) indicate that a reversion of highly overlapping endothelial cells can be induced by a conditioned medium taken from confluent endothelial cells that have adopted a monolayer configuration. This medium is currently being analyzed for the presence of various components of cellular origin which might be responsible for the induced changes in cellular morphology and growth characteristics (Greenburg, Vlodavsky, and Gospodarowicz, manuscript in preparation).

The present study presents new aspects of the pleiotropic interaction between FGF and a given cell type. This is best demonstrated by the observation that FGF can be involved in both the proliferation and differentiation of vascular endothelial cells. A similar observation has already been made for the nerve growth factor (NGF), which has been shown to control both the proliferation and differentiation of neuronal cells (21). However, in the case of NGF, because of the specific nature of its target cells, which lose their proliferative capacity early in their lifespan, the mitogenic and differentiating effects are observed at different times in the cells' lives. Our recent studies with rat pituitary cells have also demonstrated an effect of EGF on gene expression and differentiated functions (synthesis of prolactin and growth hormone) which was not associated with a mitogenic response (Johnson, Baxter, Vlodavsky, and Gospodarowicz, manuscript in preparation).

In contrast, with vascular endothelial cells, which retain their proliferative capacity throughout their lifespan, FGF can simultaneously be both a proliferative and differentiating agent. Its long-term effects on the differentiation and physiological function of the vascular endothelium should therefore not be overlooked by studying various immediate and relatively short-term responses such as changes in metabolic behavior and proliferation index.

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