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Structural and Functional Alterations in the Surface of Vascular Endothelial Cells Associated With the Formation of a Confluent Cell Monolayer and With the Withdrawal of Fibroblast Growth Factor

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Vascular endothelial cells cultured in the presence of fibroblast growth factor (FGF) divide actively when seeded at low or clonal cell densities and upon reaching confluence adopt a morphologic appearance and differentiated properties similar to those of the vascular endothelium in vivo. In this review, we present some of our recent observations regarding the characteristics (both structural and functional) of these endothelial cells and the role of FGF in controlling their proliferation and normal differentiation. At confluence the endothelial cells form a monolayer of closely apposed and nondividing cells that have a nonthrombogenic apical surface and can no longer internalize bound ligands such as low-density lipoprotein (LDL). The adoption of these properties is correlated and possibly causally related to changes in the cell surface such as the appearance of a 60,000 molecular weight protein (CSP-60); the disappearance of fibronectin from the apical cell surface and its concomitant accumulation in the basal lamina; and a restriction of the lateral mobility of various cell surface receptor sites. In contrast, endothelial cells that are maintained in the absence of FGF undergo within three passages alterations that are incompatible with their in vivo morphologic appearance and physiologic behavior. They grow at confluence on top of each other and hence can no longer adopt both the structural (CSP-60, cell surface polarity) and functional (barrier function, nonthrombogenicity) attributes of differentiated endothelial cells. Since these characteristics can be reacquired in response to readdition of FGF, in addition to being a mitogen FGF may also be involved in controlling the differentiation and phenotypic expression of the vascular endothelium.

Key words: **fibronectin, CSP-60, extracellular matrix, thrombogenic properties, low-density lipoprotein, receptor redistribution, asymmetry of cell surfaces, cell morphology, spatial configuration**

Endothelial cells constitute the inner lining (intima) of the blood vascular system and play an active role in normal hemostasis. They function as a selective permeability barrier, actively participate in the metabolism of vasoactive substances, and have a nonthrombogenic surface exposed to the bloodstream [1–6]. Abnormalities of the endothelial cell structure

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and function are therefore prominent in the pathology of a number of blood vessel diseases leading to thrombus formation or to the development of an atherosclerotic plaque. The elucidation of the factors involved in endothelial cell survival, continuity, and differentiation can be best studied using the tissue culture approach. This may shed light on characteristics that enable the endothelial layer to function as an effective permeability barrier, to resist high pressure and sheer forces, and to form a nonthrombogenic surface.

A number of laboratories [4–7] are now routinely maintaining vascular endothelial cells in culture, the only limitation being a short life-span (20–40 generations) and a requirement for a low (up to 1:8) split ratio. In contrast, the use of fibroblast growth factor (FGF) has led us to establish in culture a variety of *cloned* endothelial cell lines derived from vascular territories as diverse as fetal, neonatal, and adult vein, arteries, and heart [8, 9]. These cell lines divide with a high mitotic index when maintained at low or clonal cell densities and when they are confluent, they exhibit the morphologic and metabolic characteristics of vascular endothelial cells in vivo as expressed by the preservation of their shape and spatial organizations, non-thrombogenic apical surface, and ability to produce a basement membrane, as well as by other structural and functional properties which we are presenting here. These cell lines are indistinguishable from their in vivo counterparts in their karyotype, even after being passaged in vitro for 400 generations [1–3].

The endothelial apical surface, being located at the interface between blood and tissue, is expected to fulfill a major role in various differentiated functions of the vascular endothelium (permeability barrier, nonthrombogenicity) and could therefore differ from the basal cell surface, which functions in the production of a basement membrane to which the endothelium is firmly attached [1–3, 5, 10]. Therefore, following the interaction between cells that leads to the adoption of a monolayer configuration one might expect a reorganization of proteins in the cell surface, some of whose synthesis is turned on while that of others is turned off. This will in turn lead to the asymmetric organization of the cell surface characteristic of the vascular endothelium. In the following study, we present our observations a) on changes in the structural organization of cell surface proteins (fibronectin, CSP-60) and in surface receptor (low-density lipoprotein [LDL], concanavalin A) redistribution associated with the formation of a confluent cell monolayer, and b) on cell surface changes (protein organization, surface asymmetry, platelet-binding capacity) associated with maintaining the endothelial cells in the absence of FGF. These observations are discussed in relation to the normal differentiation of the vascular endothelium and to the protective role of the endothelial layer against an uncontrolled uptake of LDL and thrombi formation. We wish to emphasize that we have not attempted to review the pioneering and important studies of other groups working with cultured endothelial cells. Instead, we have chosen to present some of our recent studies, in particular those dealing with changes in the endothelial cell surface, as well as our overall outlook regarding the use and role of FGF in establishing long-term cultures of vascular endothelial cells that adopt the morphologic appearance and differentiated properties of the vascular endothelium in vivo.

MATERIALS AND METHODS

Cells

Bovine endothelial and smooth muscle cells were obtained from the fetal heart and the aortic arches of adult animals [8, 9]. Bovine corneal endothelial cells were obtained

from steer eyes [11]. Cells were cloned and cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, H-16), as previously described except that in some experiments a fibronectin-free serum, rather than regular calf serum, was used. For this purpose, the fibronectin present in serum was first adsorbed by affinity chromatography on gelatin-Sepharose as described [12]. Endothelial cells were passaged weekly at a 1:64 split ratio. Fibroblast growth factor (FGF, 100 ng/ml) was added every other day until the cells were nearly confluent. Similar results were obtained with confluent cultures that were continuously maintained with FGF (added every other day). Presence of factor VIII antigen and the adoption of a highly organized morphology of flattened and closely apposed cells at confluence have been constant features of all subcultures of the vascular endothelial cells [1–3, 8–10]. Cultures were used when sparse (178 cells/mm^2) or subconfluent (458 cells/mm^2), or 8–10 days after reaching confluence (713 cells/mm^2). FGF was purified, as previously described, from bovine pituitary glands [13] and bovine brains [14]. Both pituitary and brain FGF yield single bands in polyacrylamide disk gel electrophoresis at pH 4.5. Endothelial cells that were seeded in the absence of FGF and no longer maintained with FGF, lost within three passages their unique morphologic organization at confluence, became considerably larger, and grew in multiple layers.

Iodination of Cell Surface Proteins

Radioiodination of cell monolayers catalyzed by lactoperoxidase-glucose oxidase was carried out in Dulbecco's phosphate-buffered saline (PBS) in the presence of ^{131}INa (Amersham), lactoperoxidase (Calbiochem), and glucose oxidase (Sigma) as described by Teng and Chen [15]. Iodinated cells were washed five times with Ca^{2+},Mg^{2+}-free PBS and lysed in buffer containing 15% glycerol, 2% sodium dodecyl sulfate (SDS), 75 mM Tris-HCl (pH 6.8), and 2 mM phenylmethylsulfonyl fluoride and 2 mM EDTA to inhibit proteolysis. To block free sulfhydryl groups [16], 1 mM N-ethylmaleimide and 1 mM iodoacetic acid were added. The cell lysates were then boiled for 2 min to denature nucleic acids and proteins. To reduce disulfide bonds, dithiothreitol (DTT) were added to a concentration of 0.1 M before the boiling step [16].

Polyacrylamide Gel Electrophoresis

Samples containing 50,000–100,000 protein-bound cpm were applied to exponential gradient polyacrylamide slab gels with a 3% stacking gel [17]. The standards used for molecular weight determinations were [^{35}S] methionine-labeled T_4 phage proteins. For analysis in two dimentions [18], appropriate individual lanes were cut out of the first-dimension slab gel, and each lane was placed on top of a second slab gel and sealed in place with 0.1% (w/v) agarose in electrophoresis buffer. For reduction prior to the second dimension, the agarose contained 5%-mercaptoethanol. After electrophoresis, the slab gels were fixed in 7% (w/v) acetic acid, dried onto filter paper, and subjected to autoradiography on Kodak NS-2T X-ray film for 8–24 h.

Metabolic Labeling and Immunoprecipitation of Fibronectin

Sparse and confluent cultures were labeled with [^{35}S] methionine (20 h, 65 μCi/ml) in DMEM containing 0.5% calf serum and 10^{-5} M L-methionine. The tissue culture medium was then collected, the cell layer was washed, and both were analyzed by SDS polyacrylamide gel electrophoresis (PAGE) before or after immunoprecipitation with antifibronectin antiserum. For this purpose, cells were extracted with Tris-buffered saline (pH 8.0) containing 0.1% SDS, 0.5% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride (PMSF).

Insoluble material was removed by centrifugation of the medium and cell extract at 15,000g for 15 min at 4°C. Rabbit anti-bovine plasma fibronectin antiserum or nonimmune rabbit serum (10 μl) was added to 0.25 ml cell extract or to 1 ml of growth medium and after incubation for 2 h at 37°C the antigen-antibody complexes were precipitated by addition of 10 μl of goat anti-rabbit IgG antiserum (1 h at 37°C followed by 16 h at 4°C). The immunoprecipitates were washed and collected by centrifugation as described [19], dissolved in a sample buffer, and analyzed by SDS polyacrylamide slab gel electrophoresis either before or after reduction with dithiothreitol as described above.

Indirect Immunofluorescence Staining

Cells 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 5 min with 10% formaldehyde in PBS, and washed extensively with PBS. They were then incubated for 30 min with a 1:40 dilution in the same medium of rabbit anti-bovine fibronectin antiserum, generously provided by Dr. C. R. Birdwell (Scripps Research Clinic, La Jolla, California). This antiserum gave by immunoelectrophoresis one precipitin line against whole bovine plasma or serum and cross-reacted with fibronectin from several species [20]. 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-conjugated (FITC-conjugated) goat anti-rabbit IgG (Meloy Co., Virginia) in the same medium. Cultures were rinsed six times in PBS and once in distilled water and mounted for microscopy in buffered glycerol. 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.

Staining With fl-Anti LDL

All experiments were initiated after cells had been incubated for 48 h in DMEM supplemented with lipoprotein-deficient serum (LPDS). Cell cultures were incubated for 1 h at 37°C with human LDL (50–100 μg/ml), chilled, and washed briefly eight times at 4°C with phosphate-buffered saline (pH 7.4) containing 0.2% bovine serum albumin. Each culture was then incubated (1 h, 4°C) with a 1:30 dilution of fluorescein isothiocyanate-conjugated IgG fraction of rabbit anti-human LDL serum (fl-Anti LDL, Research Plus Co.) and washed eight times at 4°C. Incubations of cells at 4°C and 37°C were in DMEM containing LPDS (2.5 mg/ml), except that at 4°C the bicarbonate was replaced by 10 mM Hepes (pH 7.4). Coverslip cultures were then fixed (30 min, 22°C) with 3.7% formaldehyde in PBS and mounted for microscopy in buffered glycerol. To study the distribution of fl-Anti LDL in a single cell suspension, cells in monolayer were incubated with LDL and fl-Anti LDL as described, dissociated (2–3 min, 37°C) with a 0.03% EDTA solution, and then either fixed or immediately examined by phase contrast and fluorescence microscopy. In some experiments, fl-Anti LDL Fab fragments rather than intact IgG anti-LDL antibodies were used. Exposure of cells at 4°C to fl-Anti LDL, in the absence of an initial exposure to LDL, failed, under the indicated conditions, to stain the cells or gave at most a very slight uniform background of fluorescence. Because intact cells exclude antibody molecules,

staining of chilled cells with fluorescein-conjugated anti-LDL rather than with fluorescein coupled directly to LDL permits the selective visualization of the LDL bound to the cell surface.

In order to improve the sensitivity of the immunofluorescence staining, cell monolayers in some experiments were incubated first with LDL, then with a 1:40 dilution of rabbit anti-human LDL antiserum, and finally with fluorescein-conjugated goat anti-rabbit IgG. This procedure also heightened, especially in sparse cultures, the background of fluorescence caused by staining of LDL particles nonspecifically adsorbed to the coverslip.

Cells were incubated, under all conditions, with LDL or with fl-Anti LDL when attached to the tissue culture dish. Washings were performed at 4°C. This allowed rapid washing of the cells and greatly reduced the dissociation and internalization of the bound LDL that occurs in cells that are washed free of unbound molecules.

Staining With fl-Con A

Coverslip cultures or EDTA-dissociated cells in suspension were washed twice in PBS containing 1 mM glucose and incubated (15 min, 37°C) in the same medium with fluorescein isothiocyanate-conjugated Con A (fl-Con A, Yeda Research and Development Co., Rehovoth, Israel) at 50 μg/ml. Coverslips were then washed briefly five times, fixed (3.7% formaldehyde, 30 min, 24°C), and mounted for microscopy in buffered glycerol. Cells in suspension were washed twice (4 min × 300g) and scored for their fluorescence staining pattern either before or after fixation with formaldehyde. Staining was inhibited completely by exposing the cells to fl-Con A in the presence of 0.05 Mα-methyl-D-mannoside.

In order to avoid detection of Con A molecules which are taken into the cells, cells were also incubated with native rather than fluorescein-conjugated Con A, followed by fixation (3.7% formaldehyde, 15 mm, 24°C) and exposure to a 1:15 dilution of fl-anti-Con A (Cappel Laboratories). Fluorescence was observed with a Leitz orthoplan fluorescence microscope under epi-illumination and a planar 63/1.4 oil objective. Photographs were taken on Kodak Tri-X film using a Leitz orthomat camera.

Lipoproteins

Human LDL (1.019 density 1.063 g/cm^3) and human lipoprotein-deficient serum (density 1.21 g/cm^3) were obtained from plasma by differential ultracentrifugation [21]. Chemical modification of LDL with N,N-dimethyl-1,3-propanediamine (DMPA) was performed with ethyl-3,3-dimethylaminopropyl carbodiimide (Sigma) as described [22]. Cationized LDL was optically clear and, when compared to native LDL by electrophoresis in agarose (pH 8.6), remained in the sample well, while the native LDL migrated toward the anode, as previously reported [22]. Cationized LDL gave a precipitin line with anti-human LDL antiserum. Native and cationized LDL were iodinated using a modification of the iodine monochloride method [23] as described [24–26].

Biochemical Assays

Measurements of surface-bound (heparin-releasable) ^{125}I-LDL as well as internalization and degradation of ^{125}I-LDL by endothelial and smooth muscle cells were carried out as previously described [24–27]. Incorporation of (1-^{14}C)-acetate and (^{3}H)-oleate into free cholesterol and cholesteryl esters, respectively, was determined after extraction of the cellular lipids by thin-layer chromatography, as described [26].

RESULTS

Surface Changes Associated With the Formation of a Confluent Cell Monolayer

Immunofluorescence and metabolic labeling studies have demonstrated that in vascular endothelial cell cultures, depending on the cell density, large quantities of fibronectin can be found in the tissue culture medium, the plasma membrane of the cells, or the extracellular matrix [1–3]. Since fibronectin has been shown to be involved in cell-cell interaction and cell-substrate adhesion, we have looked for changes in its localization and surface distribution as a function of the cell density and spatial organization.

For these experiments, cells were cultured in the presence of serum that was first depleted in its fibronectin content [12, 20] to avoid detection of fibronectin derived from the calf serum. The importance of cell-cell contacts as a signal for the appearance of fibronectin was reflected by its apparent absence on endothelial cells that are not yet in contact (Fig. 1A) and its presence on cells that contact each other (Fig. 1B). In sparse and subconfluent cultures, thin fibers of fibronectin were seen along the apical cell surface as well as in the areas of cell-cell contacts (Fig. 1B). In subconfluent and not yet organized cultures the fibronectin was localized mainly in areas restricted to cell-cell contact and tended to disappear from the apical cell surfaces. Finally, little or almost no fibronectin was observed late at confluence, after the cells had adopted a closely apposed and tightly packed monolayer configuration (Fig. 1C). Occasionally, with careful focusing, the presence of scattered areas of fluorescence could be observed, and this was in most cases related to areas where the cells retracted and the extracellular matrix became exposed.

Fibronectin is a major component of the extracellular matrix produced by a variety of cell types both in vivo and in vitro [28]. Since fibronectin is resistant to treatment with low concentrations of nonionic detergents, its presence and organziation in the basal lamina can be demonstrated after the cell layer is removed with Triton X-100. Figure ID shows the immunofluorescence staining pattern of fibronectin in the extracellular matrix left on the tissue culture dish after removing a 4-week-old, confluent endothelial cell monolayer. The fibronectin is located in fibrillar structures distributed in correspondance to the organization of the matrix. Radioiodination (lactoperoxidase/glucose oxidase) of the extracellular matrix followed by SDS slab gel electrophoresis revealed (Fig. 2A) that the fibronectin identified by double immunoprecipitation (Fig. 2B) is the major component of the endothelial basal lamina and is extensively disulfide-bonded into dimers and larger aggregates that hardly penetrates the running gels. Reduction with DTT greatly decreased the amounts of these high-molecular-weight complexes and yielded a major band that comigrated with a fibronectin monomer (Fig. 2F). Sparse and subconfluent cultures deposited little or no extracellular material onto the tissue culture dish. These results demonstrate that concomitant with the formation of a confluent vascular endothelial cell monolayer, fibronectin is no longer found on the apical cell surfaces but rather becomes a major component of the extracellular matrix and forms a meshwork of disulfide-bonded fibrils that are closely associated with the basal cell surface. Endothelial cells therefore differ from fibroblasts [28] and smooth muscle cells [29], where the fibronectin not only appears underneath the cells but is also found over the entire cell surface regardless of the degree of confluence. This unique polarity in the production of fibronectin by the confluent vascular endothelium could be indirectly related to the nonthrombogenic properties of the vascular endothelium and reflect a rearrangement of cell surface proteins as cells become confluent and stop dividing.

The production of fibronectin by confluent endothelial cells was further studied by

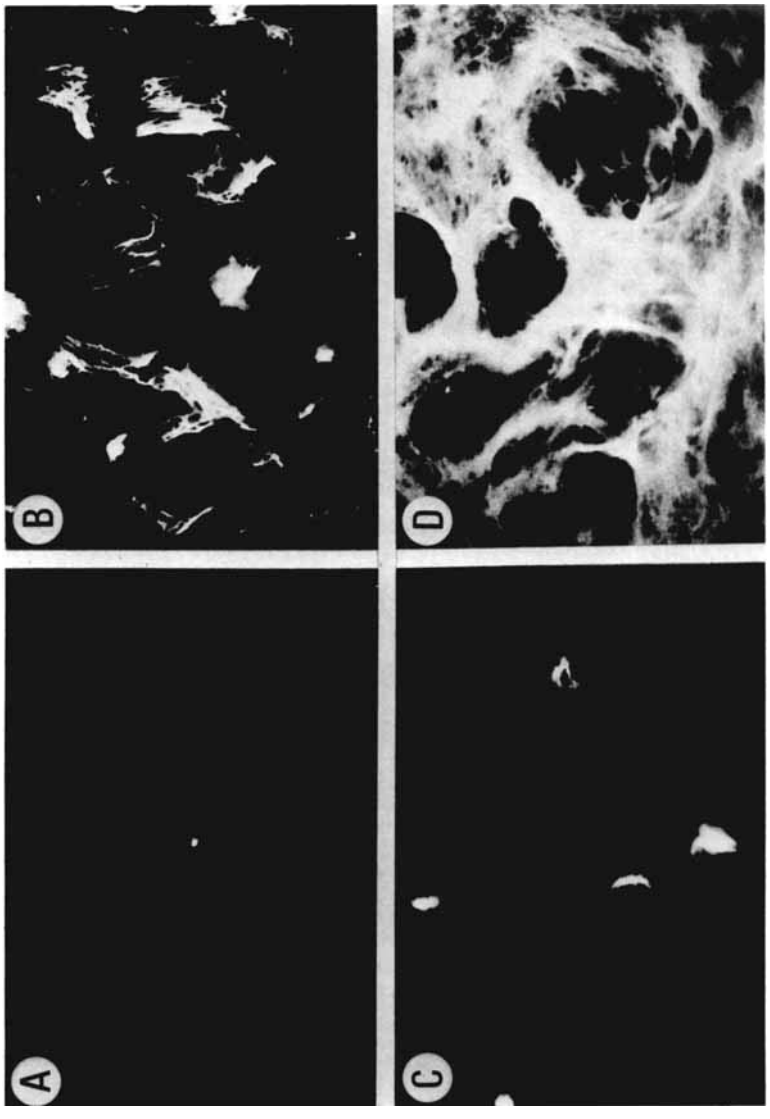

Fig. 1. Indirect immunofluorescence localization of fibronectin in vascular endothelial cell cultures. A) Staining of cells prior to the formation of cell-cell contacts shows the absence of fibronectin. B) Staining of a subconfluent culture showing the distribution of fibronectin on the apical cell surface and preferentially in the areas of contact between cells. C) Staining of a 4-week-old confluent culture with little or no detectable fluorescence. D) Staining of the extracellular matrix left after removal of the confluent cell layer with Triton X-100 (0.5%, 5 min at 37°C). All photomicrographs were at × 400.

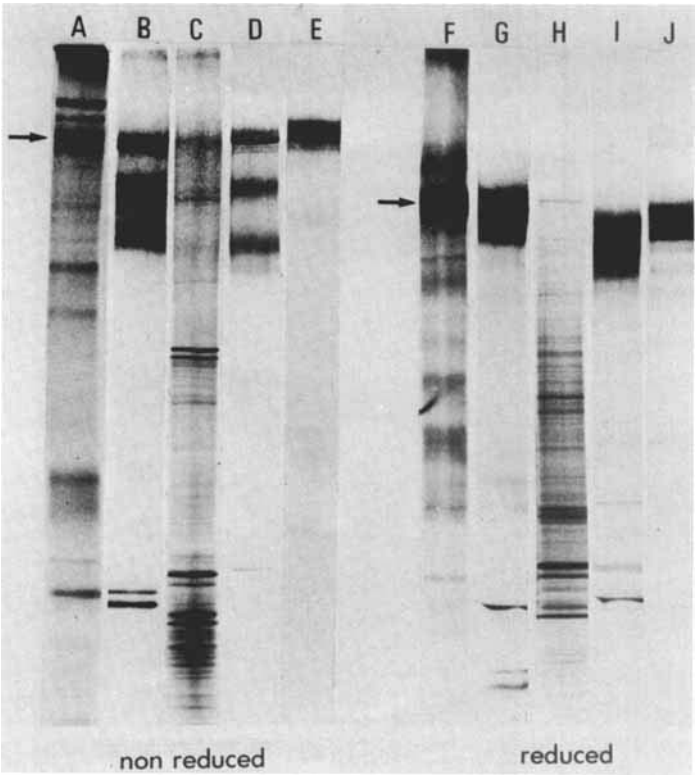

Fig. 2. SDS polyacrylamide gel electrophoresis of the extracellular matrix produced by a confluent vascular endothelial cell monolayer. Vascular endothelial cells maintained in culture for 2 weeks after reaching confluence were treated with 0.5% Triton X-100 to remove the cell layer, and the extracellular matrix thus exposed was then iodinated with iodine 131 and lactoperoxidase. Alternatively, the culture was first incubated with [^{35}S] methionine (65 μCi/ml, 20 h), the medium was collected, and the cell layer was removed with Triton X-100 to expose the underlying basement membrane. The labeled basement membranes were either dissolved in a sample buffer (lanes A, F) or subjected to a double immunoprecipitation with antifibronectin (lanes B, G, and D, I) (rabbit anti-bovine plasma fibronectin followed by goat anti-rabbit IgG, as described under Materials and Methods). Samples were analyzed by a gradient (4.5–16%) polyacrylamide gel electrophoresis either before (lanes A–E) or after (lanes F–J) reduction with 0.1 M DTT. A,F) ^{131}I-labeled vascular endothelial basement membrane. Total extract in 2% SDS (sample buffer). B,G) ^{131}I-labeled basement membrane extracted with 0.5% Triton X-100 and 0.1% SDS and subjected to a double immunoprecipitation with antifibronectin. C,H) [^{35}S]-methionine-labeled vascular endothelial basement membrane. Total extract prior to immunoprecipitation. D,I) [^{35}S] methionine-labeled basement membrane after a double immunoprecipitation with antifibronectin. E,J) [^{35}S] methionine-labeled growth medium precipitated by antifibronectin. Arrows mark the positions of fibronectin dimers (460K, nonreduced samples) and monomers (230K, reduced samples). Iodinated cultures were dissolved in electrophoresis sample buffer (2% SDS) (lanes A, F) to obtain the total protein iodination pattern or in Tris-buffered saline containing 0.1% SDS and 0.5% Triton X-100 (lanes B, G) to allow the immunoprecipitation reaction. In the latter case, insoluble material was first removed by centrifugation, and this may account for the lower amounts or absence of large fibronectin aggregates in the immunoprecipitates. [^{35}S] methionine-labeled cultures were extracted only with the immunoprecipitation medium (0.1% SDS, 0.5% Triton X-100), and the samples were centrifuged before being applied for either gel electrophoresis or immunoprecipitation. Proteolytic degradation of fibronectin seemed to have taken place during the incubation required for immunoprecipitation (lanes B, G and D, I).

exposing cultures to [^{35}S] methionine 2 weeks after they had reached confluence. The newly synthesized fibronectin was then specifically precipitated by a double antibody technique from the growth medium (Fig. 2E, J), cell extract, and the extracellular matrix underlying the confluent cell monolayer (Fig. 2D, I). These experiments have shown that most of the fibronectin produced at a late stage of confluence no longer remains as a cellular component but is instead largely secreted into the tissue culture medium and, to a lesser extent, toward the basement membrane underlying the cell monolayer.

CSP-60 and the Formation of a Confluent Cell Monolayer

Cell surface components are involved in cell-cell interactions and have been shown to undergo structural changes in response to cell contacts [30, 31]. Since cultured vascular endothelial cells mimic their in vivo counterparts in their two-dimensional organization, asymmetry, and barrier function, these cells provide a system with which to study whether changes in the cell surface correlate with the ability of the endothelial cells to adopt at confluence the morphologic appearance of a highly organized monolayer composed of closely apposed and flattened cells. To study whether surface components are involved in the adoption of such a morphology and, if so, what these components are, we have looked for surface proteins that are affected by changes in the cell density or by dissociation and formation of cell-cell contacts. Density-dependent changes in the cell surface were studied by use of the lactoperoxidase catalyzed iodination to label in sparse, subconfluent, and confluent cultures the apical cell surface of vascular endothelial cells. The labeled proteins were analyzed by gel electrophoresis and their pattern was compared with that of such cell types as vascular aortic smooth muscle cells, which in contrast to the vascular endothelium grow at confluence in multilayers (Fig. 3). We also studied whether changes in the appearance of various surface proteins could be induced by disorganization and reorganization of a confluent endothelial cell monolayer (Figs. 4 and 5). Our results indicate a clear correlation between the formation of a highly organized endothelial cell monolayer and the appearance of a 60K molecular weight component which has been named CSP-60 [32]. This correlation is based on the following findings. a) Under various experimental conditions, the formation of a closely apposed and highly organized endothelial cell monolayer was associated with the appearance of CSP-60 as a major cell surface component susceptible to iodination by lactoperoxidase. This occurred under normal conditions, ie, as soon as the culture adopted the configuration of a confluent monolayer composed of tightly packed and cuboidal cells (Fig. 3A, E) or in reconstituted monolayers (Fig. 5B, C, F, G, K, L) derived from cultures that were first dissociated into single cells by urea (Fig. 4D), trypsin, or EDTA. b) In the various cultures studied thus far, CSP-60 was not detected when cells were sparse (Fig. 3B, F and Fig. 5D, H, M) or when no contact between the cells existed (Fig. 5E, J). Likewise, CSP-60 was not present at a subconfluent density (Fig. 6B), when cells contacted each other but were still elongated, overlapped each other, and were not yet organized in a tightly packed cuboidal manner (Fig. 4B). c) Cells which grow in multiple layers at confluence (ie, smooth muscle cells) contained no CSP-60 even after being maintained at confluence for an extended period of time (Fig. 3C, G). d) CSP-60 was not present in a disorganized cell monolayer (Fig. 5E, J) or up to 72 h after reseeding the confluent cells at a low density, nor could it be found in sparse cells that were pooled and reseeded at a high density (Fig. 6D) to yield a confluent but unorganized endothelial culture (Fig. 4H).

After disruption of an already formed cell monolayer, both the appearance of CSP-60 and the adoption of a monolayer configuration required a short time (2–5 h). In comparison,

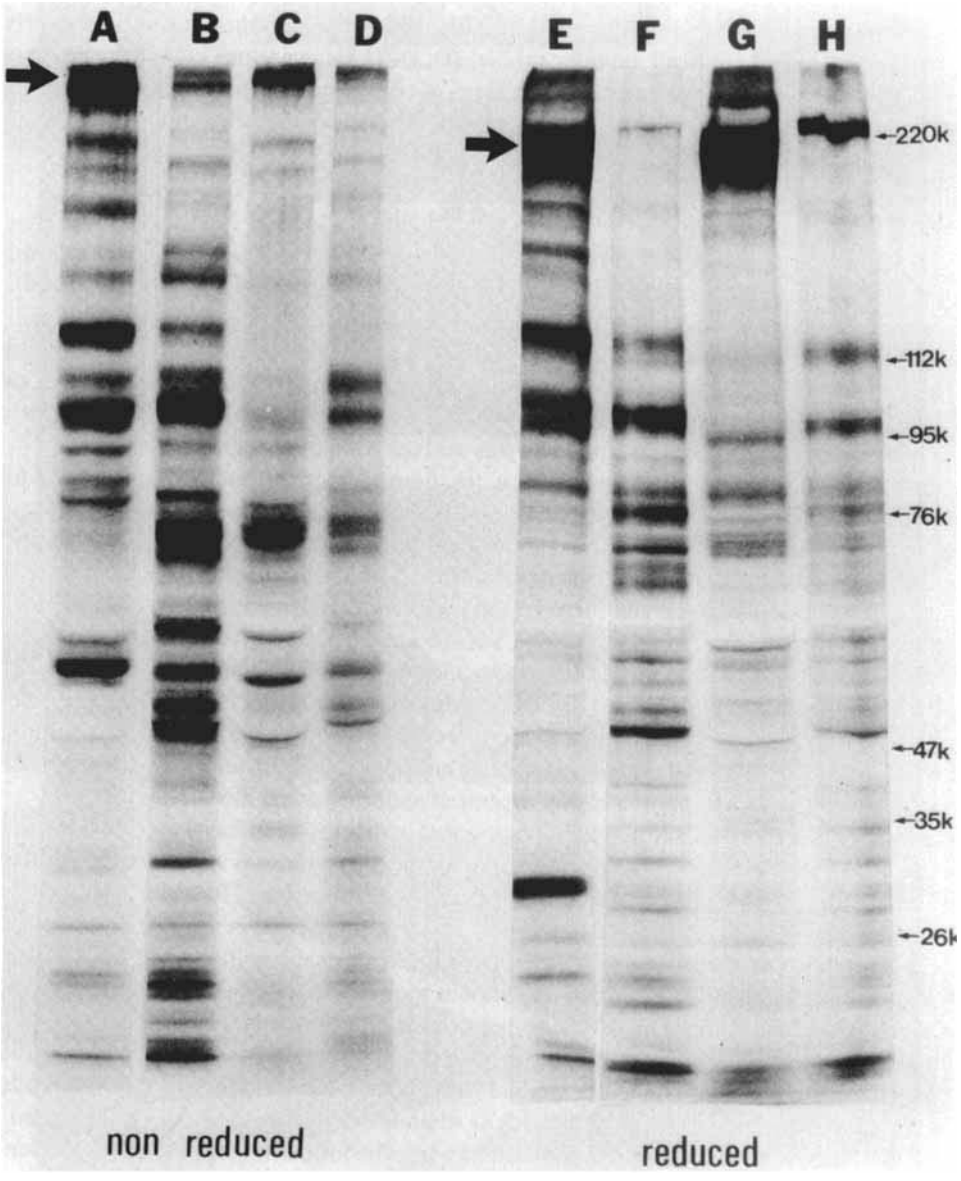

Fig. 3. SDS-polyacrylamide gel electrophoresis of lactoperoxidase-iodinated sparse and confluent cultures of vascular endothelial and smooth muscle cells. Washed cells were radioiodinated, lysed, and analyzed by a gradient (6.5–15%) PAGE either before (lanes A–D) or after (lanes E–H) reduction with 0.1 M DTT. A,E) Confluent monolayers of bovine aortic endothelial cells. B,F) Sparse culture of bovine aortic endothelial cells. C,G) Confluent culture of bovine aortic smooth muscle cells. D,H) Sparse culture of bovine aortic smooth muscle cells. Gels were standardized with T_4 phage [^{35}S] methionine-labeled proteins, and arrows mark the positions of fibronectin and CSP-60.

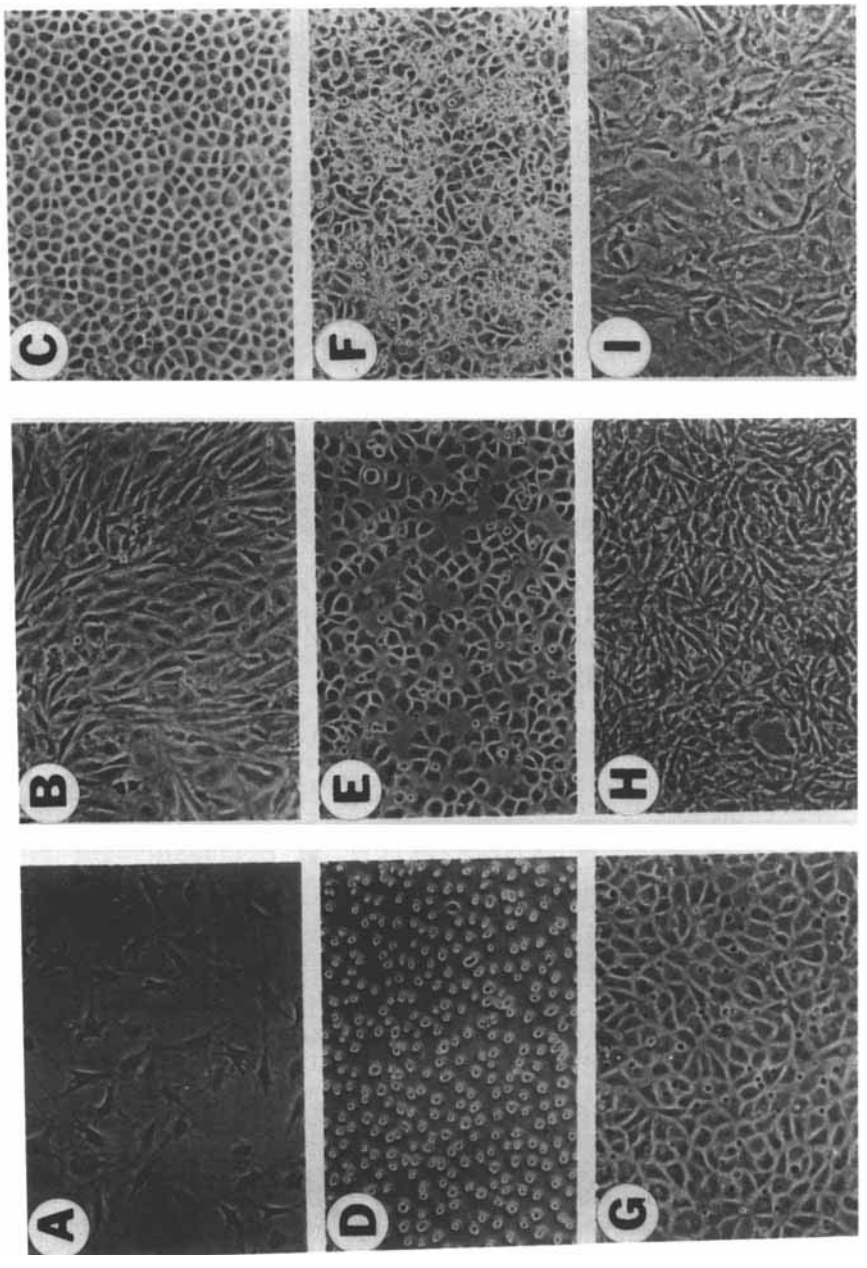

Fig. 4. Phase contrast micrographs of disorganized and reorganized monolayers of vascular endothelial cells. A) Sparse, actively growing culture, two days after seeding. B) Subconfluent culture 5 days after seeding. C) A confluent cell monolayer, 7 days after reaching confluence. D) A confluent cell monolayer treated with urea (1 M in DME, 1 h, 37°C). E) Urea-treated cells at an intermediate stage of reorganization, 1 h after washing the urea out and incubating the culture under growth conditions. F) Urea-treated cells seeded at high density and observed after 5 h. The excess of cells remain round, and do not adhere or grow on top of the attached cells. G) Urea-treated cells seeded at a high cell density and observed 24 h later after washing the excess of cells. H) Cells from a sparse culture that were pooled together, reseeded at a high density, and observed 48 h later. A similar morphology was adopted by the cultures described under E,F,G, and H when calf serum free of fibronectin, rather than normal calf serum, was used. I) Endothelial cells maintained for three passages in the absence of FGF.

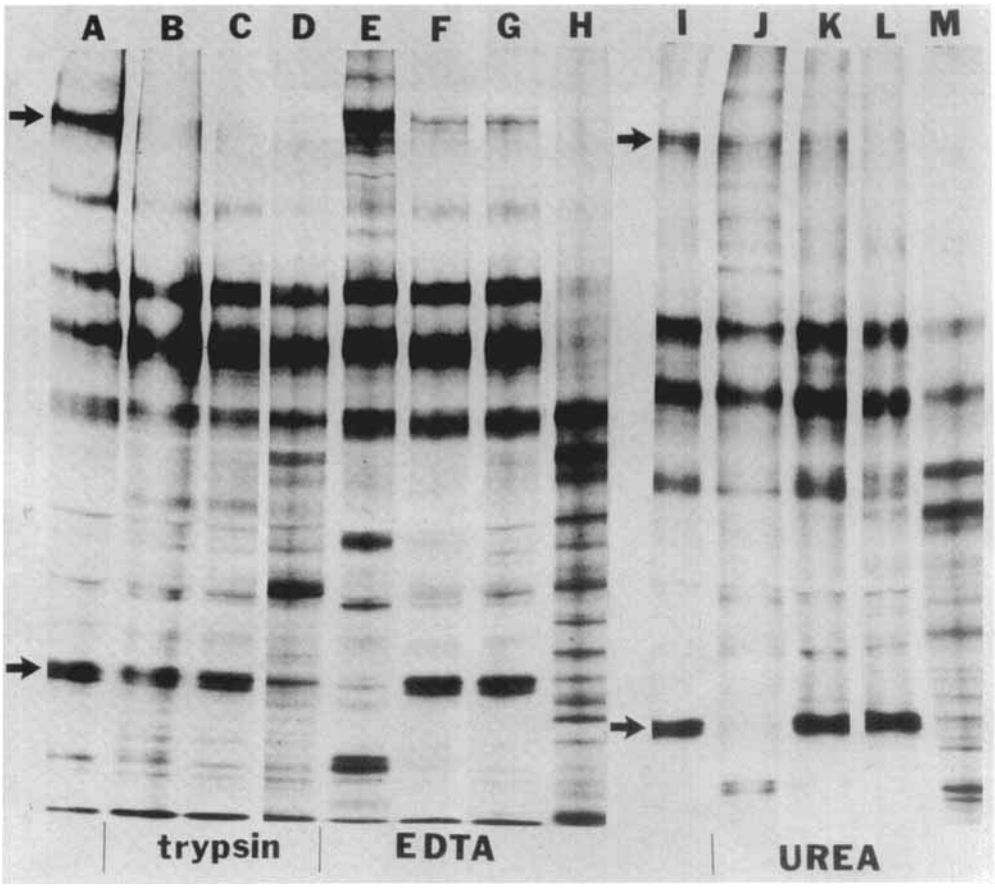

Fig. 5. Appearance of CSP-60 and fibronectin after disorganization and a subsequent reorganization of a confluent cell monolayer. Confluent endothelial monolayers were treated with either trypsin (0.05% Gibco, 3 min, 37°C), EDTA (0.03% in Ca^{2+}, Mg^{2+}-free PBS, 30 min, 37°C), or with urea (1 M in DME, 1 h, 37°C) to dissociate cell-to-cell contacts. The disruptive agent was then washed out and the cells were allowed to reorganize in DME containing a fibronectin-depleted serum on the same plate or reseeded at a high or low density. Disorganized and reorganized cultures were iodinated and analyzed by PAGE (a 6–16% gradient) after the samples were reduced with DTT. A) Confluent endothelial cell monolayer. B) Confluent culture that was first trypsinized into single cells and then washed and incubated in the same plate for 12 h under growth conditions to readopt its original monolayer configuration. C) Cells 12 h after trypsinization and seeding at a high density. The cells fully adopt a monolayer organization and show the presence of CSP-60, but little or no fibronectin. D) Cells 12 h after trypsinization and seeding at a low density. E) Confluent culture treated with EDTA and labeled when the cells detached from each other. CSP-60 is now only slightly or no longer exposed for iodination. F) Confluent culture that was first dissociated by EDTA and then incubated for 5 h under growth conditions to readopt its original morphology. G) Cells 12 h after EDTA dissociation and seeding at a high density. H) Cells 12 h after EDTA dissociation and seeding at a low density. I) Confluent cell monolayer. J) Confluent culture treated with urea and labeled when the cells appeared as single round spheres. K) Confluent culture that was first treated with urea and then allowed to reorganize by a 3-h incubation under growth conditions. L) Cells 24 h after exposure to urea and reseeding at a high density. M) Cells 24 h after treatment with urea and reseeding at a low density. Arrows mark the positions of fibronectin and CSP-60.

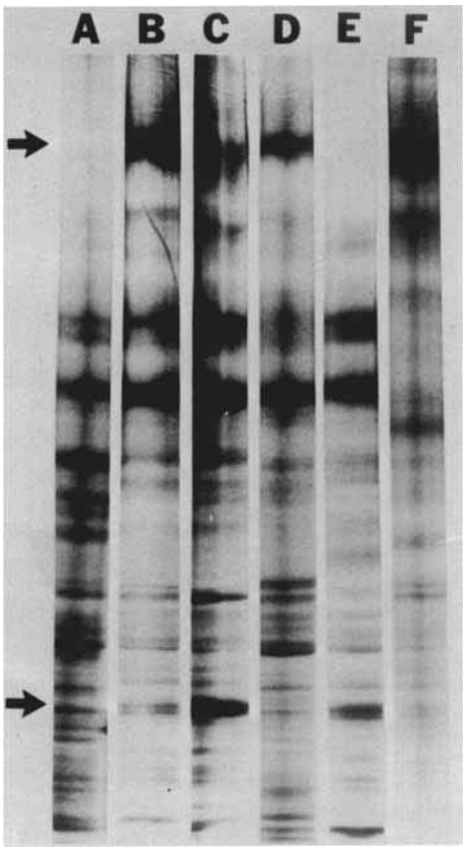

Fig. 6. Appearance of fibronectin and CSP-60 in organized and nonorganized cultures of endothelial cells. Endothelial cultures were labeled with ^{131}I and lactoperoxidase and samples applied onto a gradient (6–16%) polyacrylamide slab gel after reduction with 0.1 M DTT. A) Sparse, actively growing endothelial cells. B) Subconfluent endothelia culture. C) Confluent highly organized cell monolayer. CSP-60 appears as a major band; fibronectin is detected in small quantities compared to subconfluent cultures. D) Cells derived from sparse, actively growing cultures reseeded at a high density and iodinated 48 h later. E) Cells 12 h after trypsinization of a confluent monolayer and reseeding at a high density. F) A confluent but unorganized endothelial culture maintained for three passages in the absence of FGF. Large amounts of fibronectin and little or no CSP-60 can be detected. Arrows mark the positions of fibronectin and CSP-60.

no less than 72 h were required for actively growing cultures to acquire a similar morphology after reaching confluence. This result and the fact that 10^{-4} M cycloheximide did not prevent the reappearance of CSP-60 in a monolayer which was first disrupted and then allowed to reorganize suggest that in cells which have already reached the stage of a confluent monolayer CSP-60 is reexposed rather than resynthesized during the reorganization of the cells into a monolayer.

CSP-60 has been identified in confluent cell monolayers of all the vascular endothelial cell types studied so far, regardless of their origin or age of their donor (Fig. 7). These include endothelial cells from the bovine fetal (lanes C and c), calf (lanes G and g), and adult (lanes D and d) aortic arches; pig aortic arch (lanes B and b); fetal bovine heart (lanes E and e); bovine umbilical vein (lanes F and f); and bovine pulmonary artery (lanes A and a).

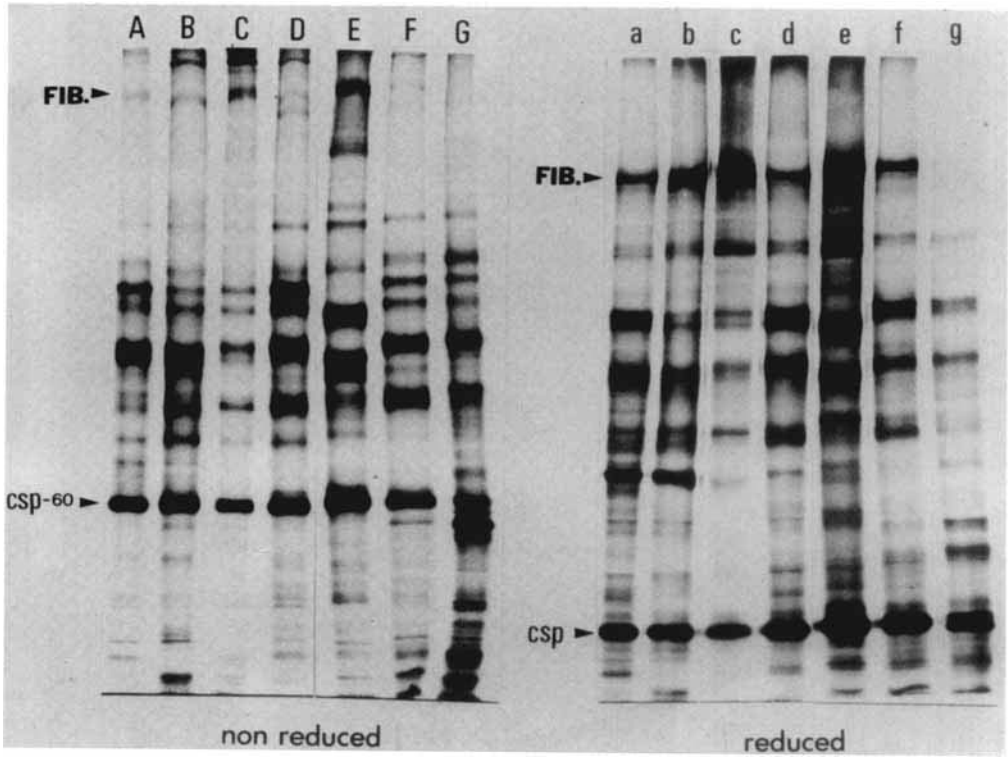

Fig. 7. Appearance of CSP-60 in various types of cultured vascular endothelial cells. Vascular endothelial cells of various origins were obtained, cloned, and maintained in culture as described [8, 9]. Confluent cell monolayers (5–7 days after reaching confluence) were radioiodinated (lactoperoxidase/glucose oxidase) and analyzed by a gradient (5–16%) PAGE before (lanes A–G) or after (lanes a–g) reduction with 0.1 M DTT. Vascular endothelial cells of the following origins were studied: A,a) Bovine pulmonary artery; B,b) adult pig aorta; C,c) fetal bovine aorta; D,d) adult bovine aorta; E,e) fetal bovine heart; F,f) bovine umbilical vein; and G,g) calf bovine aorta. Arrows mark the positions of fibronectin (460K and 230K) and CSP-60 (60K and 30K) before and after reduction of the samples with DTT, respectively.

In order further to characterize CSP-60, we have looked for its electrophoretic mobility before and after reduction with DTT. The results (Figs. 3, 7, and 8) indicate that nonreduced samples contained a 60K component that is characteristic of confluent endothelial cell monolayers and that, after reduction, was missing from its original position and yielded a major band of an apparent molecular weight of about 30K. The dimeric disulfide-bonded nature of CSP-60 was further demonstrated on two-dimensional gels, nonreduced in the first dimension and reduced in the second [18]. Samples derived from confluent vascular endothelial cell monolayers (Fig. 8B) revealed a major, off-diagonal spot that had an apparent molecular weight of about 30K and which, at the first, nonreduced dimension, migrated as a 60K component. Likewise, a similar off-diagonal spot was observed with corneal endothelial cells (Fig. 8E), which at confluence are as organized as vascular endothelial cells (1–3). There were either small amounts of or no such 30K components in samples derived from either sparse, actively growing endothelial cells (Fig. 8C) or from sparse and confluent (Fig. 8F) cultures of smooth muscle cells. Control gels, running either

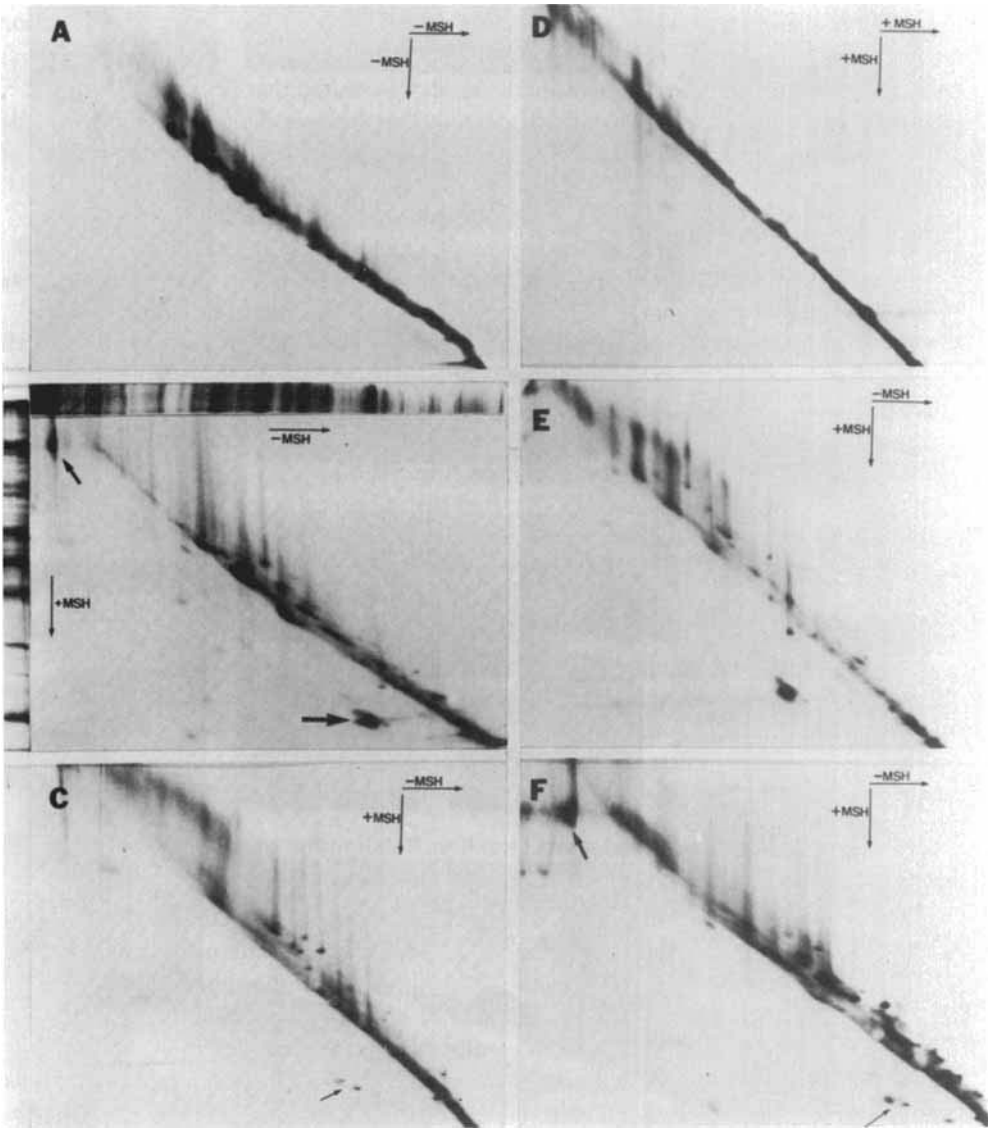

Fig. 8. Two-dimensional gel analysis of lactoperoxidase-iodinated cells. Cultures were iodinated and the cells lysed and harvested under nonreducing conditions. Aliquots were run on a gradient (6.5–16%) polyacrylamide slab gel, and the gel was cut into appropriate narrow strips that were each then placed at the top of another 6.5–16% slab gel. A reducing agent (5% MSH, 2-mercaptoethanol) was present, as shown. A,D) Confluent vascular endothelial cells. A) Both dimensions without reduction. D) Both dimensions with reduction. No off-diagonal spots are present. B) A confluent monolayer of vascular endothelial cells. Arrows mark the position of fibronectin (upper left) and CSP-60 (lower right). C) Sparse, actively growing vascular endothelial cells. Note the absence of fibronectin and CSP-60. E) A confluent corneal endothelial cell monolayer. CSP-60 appears as a major off-diagonal spot. Fibronectin is only slightly or not at all exposed to the lactoperoxidase-catalyzed iodination F) Confluent vascular smooth muscle cells. Note the absence of CSP-60 and the presence of fibronectin.

nonreduced or reduced in both directions, showed no such off-diagonal spots, proving that the appearance of the low-molecular-weight, off-diagonal spot was caused by the reduction step between the two stages of electrophoresis.

The results of these experiments have also demonstrated that fibronectin is not essential for the formation of a highly organized cell monolayer. This conclusion is based on the following observations: a) High amounts of fibronectin were detected in subconfluent but not yet organized endothelial cultures (Fig. 6B) as well as in sparse (Fig. 3D, H) and confluent (Fig. 3C, G) cultures of vascular smooth muscle cells that form multiple layers at confluence; b) Following dissociation of a highly organized vascular endothelial cell monolayer with EDTA, urea, or trypsin and upon removal of the disruptive agent, the cultures resumed their normal configuration within a 2- to 5-h incubation in a fibronectin-free medium despite the partial or nearly total removal of fibronectin from the cell surface (Fig. 5B, F, K). Likewise, when endothelial monolayers were trypsinized and the cells then seeded at a high density in a medium containing no fibronectin, the cultures resumed, within 5–12 h, their original morphology (Fig. 4F, G), despite the removal of fibronectin from the cell surface by trypsin (Fig. 5B, C).

These results indicate that CSP-60, rather than fibronectin, correlates with the formation of a confluent, highly organized cell monolayer, so that its exposure only in cells that adopt such a morphology might play an essential role in the control of contact response phenomena. CSP-60, however, was not required for substrate adhesion and flattening of cells, since it was missing from cells that adhered and spread perfectly well after first disrupting a confluent monolayer and seeding the cells at a low density (Fig. 5D, H, M). These results also suggest that in confluent cultures fibronectin serves mainly as a glue by which the cells are attached to their substratum, since, unlike CSP-60, which is only present as a surface protein, fibronectin is secreted in large quantities and is a major component of the basement membrane produced by endothelial cells.

Restriction of Surface Receptor Lateral Mobility: Relationship to a Protective Fuction of the Endothelium Against an Uncontrolled Uptake of LDL

In previous studies [24–26] we have demonstrated that, as in other cell types examined [33], actively growing vascular endothelial cells bind, internalize, and degrade LDL via a receptor-mediated pathwasy which regulates the synthesis of cholesterol, the formation of cholesterol esters, and the number of surface receptor sites for LDL [24–26]. In contrast, once the growing cells reach confluence and form a cell monolayer, they can no longer internalize the LDL and hence exhibit no lysosomal degradation and its associated regulatory biochemical responses, even though the cells bind the lipoprotein at high-affinity receptor sites [24–26]. This inhibition of internalization, but not of binding, of LDL is due to the formation of highly organized cell monolayer rather than to the inhibition of cell proliferation at confluence, since it was reversed by disrupting the cell monolayer under conditions which did not stimulate cell division [24, 25]. Furthermore, when incubated with cationized LDL rather than with native LDL, the confluent cell monolayers exhibited lysosomal degradation of the lipoprotein as well as the subsequent regulatory effects on cellular cholesterol metabolism, indicating no defect beyond the internalization step [26].

Receptor redistribution is involved in the internalization of various ligand-receptor complexes by various cell types [34–37]. It has also been shown that endocytosis is associated with a directional lateral movement of certain cell surface lectin receptors from their original random distribution into specific membrane regions [38]. These observations led us to postulate that in vascular endothelial cells, as opposed to fibroblasts and vascular

smooth muscle cells [39], a certain degree of surface receptor lateral mobility is required for the internalization of LDL. If this were the case, it would be expected that surface receptor lateral mobility in confluent endothelial cell monolayers should be severely restricted in comparison to that of endothelial cells in sparse culture.

Using fluorescein-conjugated anti-LDL (fl-Anti LDL) and fluorescein-Con A (fl-Con A), we have examined the binding and distribution of LDL and Con A molecules in sparse and confluent cultures of aortic endothelial and smooth muscle cells. Our results demonstrate that both LDL receptor and Con A receptor mobility are indeed subject to regulation by changes in the density and organization of endothelial cell cultures. In contrast, vascular smooth muscle cells do not display such density-dependent membrane changes [40].

Cells attached to the tissue culture dish. A nonrandom, splotchy to minicap-like fluorescence pattern was observed in sparse, substratum-attached, endothelial cells that were first incubated with LDL at 37°C and then chilled, washed, and exposed at 4°C to either fl-Anti LDL (Fig. 9A) or to rabbit anti-human LDL followed by fl-anti-rabbit IgG (Fig. 9B). As shown in Table I, these cells also exhibited an active uptake and lysosomal degradation of LDL. In contrast, highly confluent cell monolayers that bind but no longer internalize or degrade LDL (Table I) showed a random, although aggregated, distribution of fluorescence over the entire cell surface under the same conditions (Fig. 9D). A similar randomly distributed staining pattern was obtained with sparse endothelial cells that were preincubated with LDL at 4°C, rather than at 37°C (Fig. 9C), or that were first fixed with formaldehyde to inhibit the internalization of LDL (not shown). These results demonstrate that in actively growing, but not in confluent endothelial cell monolayers the bound LDL molecules are capable of a temperature-dependent lateral movement in the membrane plane which allows them to form large aggregates. Smooth muscle cells, unlike endothelial cells, grow on top of each other at confluence and show a similar degree of LDL internalization and degradation when maintained at either low or high cell densities (Table I) [24–26]. Accordingly, the present experiments revealed no difference between sparse and confluent cultures of smooth muscle cells, which, at both densities, showed a highly aggregated but randomly distributed anti-LDL fluorescence staining after exposure to LDL at 37°C (Fig. 9E). In each of these experiments, cells were incubated with the fluorescent antibodies at 4°C and most of the fluorescence could be removed afterwards by incubation with pronase (3 μg/ml, 30 min, 37°C), thereby demonstrating the surface localization of the LDL molecules thus detected.

Results similar to those observed with LDL were obtained when both endothelial and smooth muscle cultures were exposed to fl-Con A. In sparse cultures of endothelial cells incubated (15–20 min, 37°C) with fl-Con A, the Con A binding sites were drawn into large aggregates to form a "nuclear cap" at the center of the cells (Fig. 9F, G), thereby indicating the occurrence of Con A internalization already within 15–20 min. No such perinuclear caps, but rather a uniform distribution of fluorescence over the entire cell surface, was obtained by fixing the cells before Con A coating (Fig. 9H). In contrast to sparse endothelial cultures, confluent cell monolayers showed a random, slightly aggregated but mostly peripheral fluorescence staining pattern over the entire cell surface area (Fig. 9I). Capping by Con A was not induced in smooth muscle cells, and as already observed with fl-Anti LDL, both sparse (Fig. 9J) and confluent cultures showed, under the same conditions, a random distribution of fl-Con A. Similar results (capping under sparse conditions and a uniform distribution at confluence) were obtained when native Con A molecules and fluorescein-conjugated anti-Con A antibodies were used instead of fl-Con A, except that little or no internalization of fluorescence was observed in the latter case.

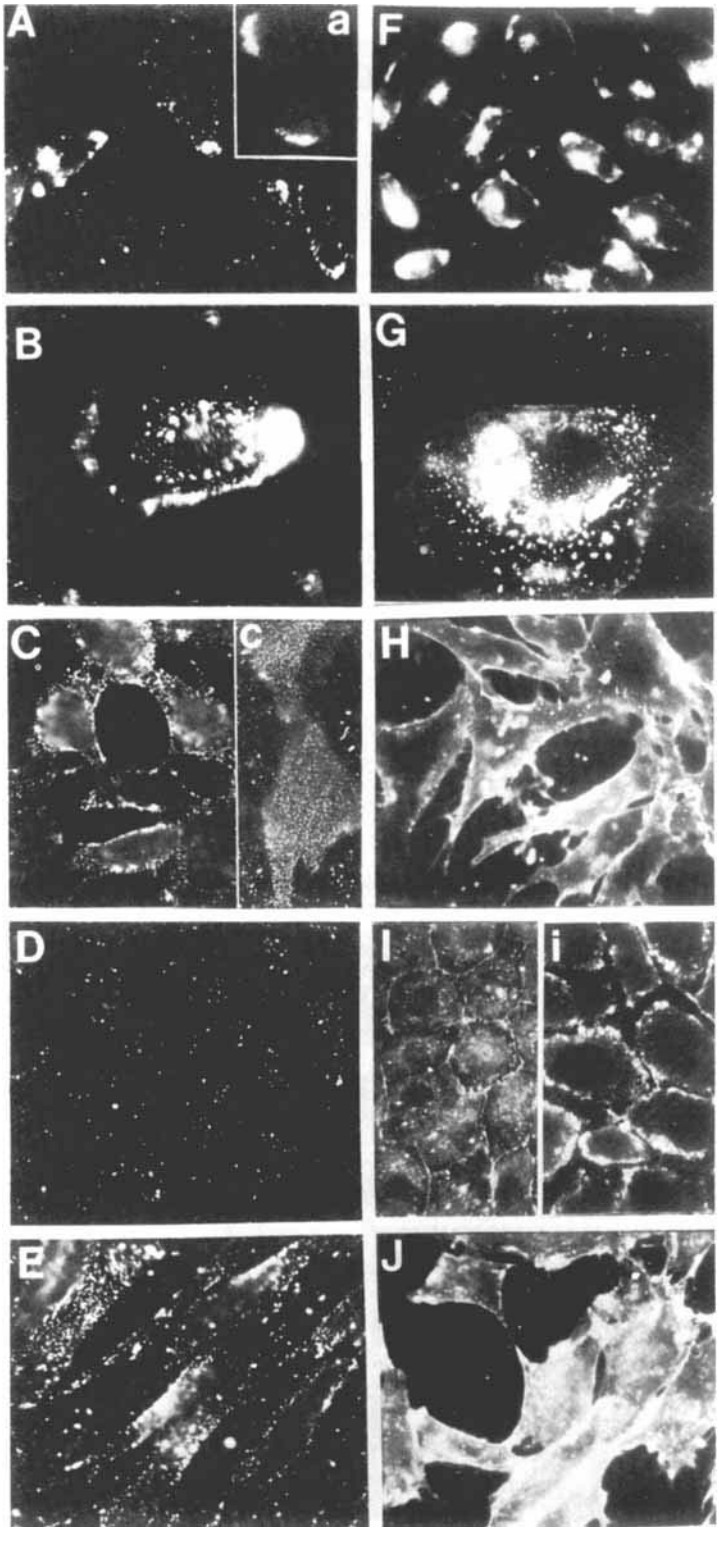
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TABLE I. Expression of the LDL Pathway in Sparse and Confluent Cultures of Vascular Endothelial and Smooth Muscle Cells

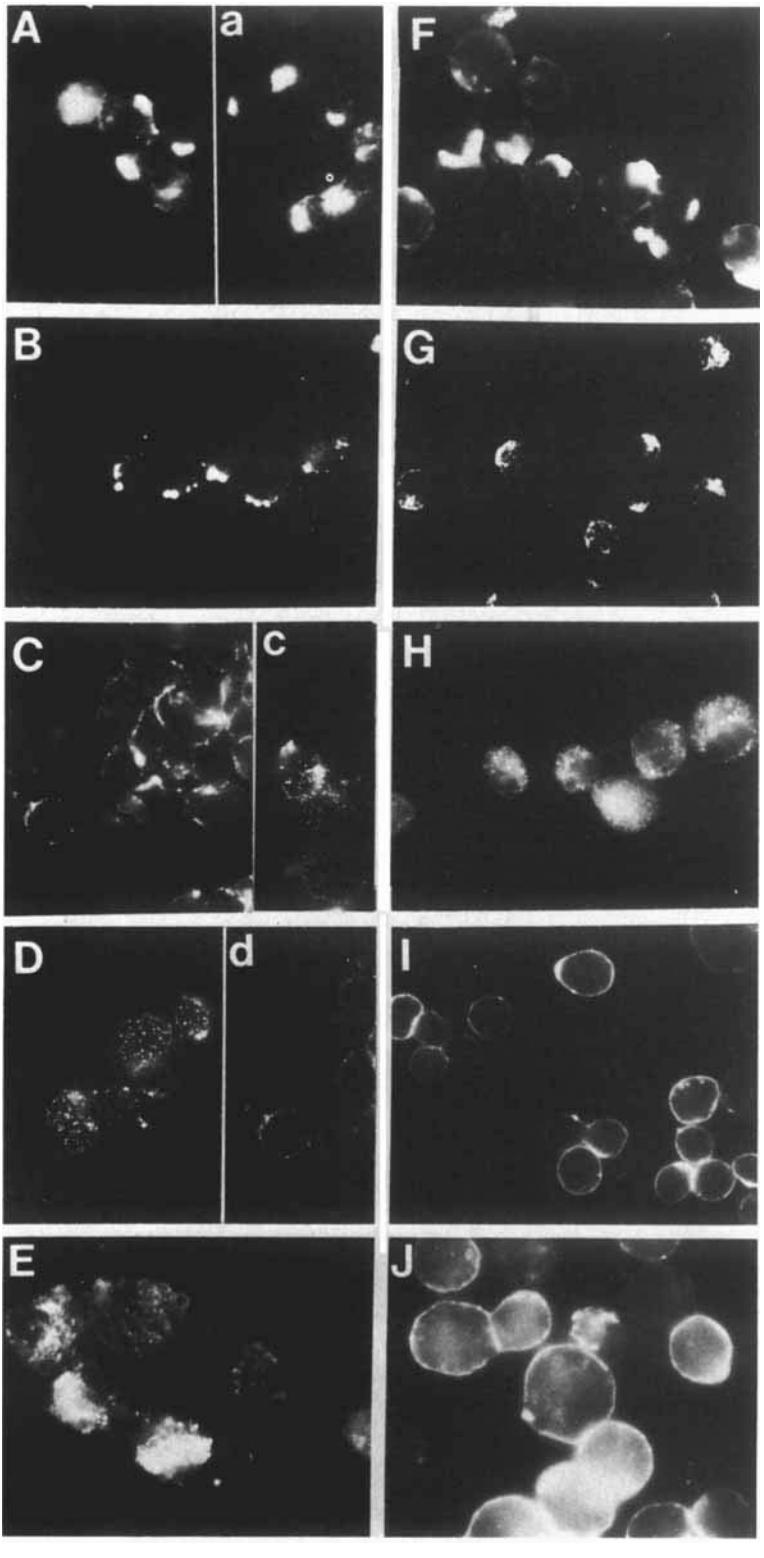
	Endothelial cells		Smooth muscle cells	
	Sparse (84 μg protein per dish)	Confluent (270 μg protein per dish)	Sparse (119 μg protein per dish)	Confluent (407 μg protein per dish)
^{125}I-LDL binding (ng/mg protein)	128	60	91	69
^{125}I-LDL internalization (ng/mg protein)	1,233	113	546	436
^{125}I-LDL degradation (ng/mg protein/6 h)	2,754	68	2,317	1,566
^{125}I-cationized LDL degradation (ng/mg protein/6 h)	3,651	1,687	1,849	1,490
Suppression of free cholesterol synthesis (96 inhibition)	73	10	89	90
Activation of cholesteryl ester formation (fold increase)	12.5	1	9	8

Data are mean values obtained from 3–5 experiments carried out under the following conditions. Sparse and confluent cultures of bovine aortic vascular endothelial and smooth muscle cells were preincubated for 48 h in growth medium containing LPDS (4 mg/ml), washed, and incubated for 2.5 h at 37°C with ^{125}I-LDL (50 μg/ml) to measure specific binding (accessible for heparin release) and internalization (heparin-resistant) of LDL [24, 27]. Cultures were also incubated (6 h, 37°C) with either native ^{125}I-LDL (20 μg/ml) or ^{125}I-cationized LDL (7.5 μg/ml) to determine the amounts of acid-soluble lipoprotein degradation products released into the medium [24, 26]. To study the regulation of cholesterol synthesis and esterification, cultures in LPDS medium were incubated (5 h and 12 h, respectively) with or without native LDL (100 μg/ml) and then with either (1-^{14}C)-acetate or (^{3}H)-oleate, respectively. Cells were then washed, harvested, and extracted to determine the content of labeled cholesterol and cholesteryl oleate as described [26].

Fig. 9. Cell surface distribution of LDL and Con A receptor sites in sparse and confluent vascular endothelial and smooth muscle cells attached to coverslips. Coverslip cultures of sparse and confluent bovine aortic endothelial and smooth muscle cells were labeled either with fl-Anti LDL or with anti-LDL and fl-anti-rabbit IgG, after first being incubated with LDL (A–E). Coverslips were also incubated directly with fl-Con A (F–J) and mounted for fluorescence microscopy as described under Materials and Methods.

A–E) Cell surface distribution of receptor sites for LDL. A,B) Sparse endothelial cultures exposed to LDL and then to fl-Anti LDL (A,a) or successively to LDL, rabbit anti-LDL, and fl-goat anti-rabbit IgG (B) as described. The LDL receptor sites are segregated into large aggregates (minicaps) located primarily in one pole of the cell. a) Cells that were rounded up during the incubations but still remained attached to the substrate. c) Sparse endothelial cells that were incubated with LDL at 4°C rather than at 37°C, washed, and stained with fl-Anti LDL at 4°C. A random distribution of fluorescence in small clusters. D) Highly confluent and contact-inhibited endothelial cells after preincubation with LDL at 37°C and with anti-LDL and fl-anti-IgG at 4°C. The LDL receptor sites are distributed in small clusters over the entire cell surface area. E) Sparse culture of smooth muscle cells treated with LDL, anti-LDL, and fl-anti-IgG as in D. A random, uniformly distributed LDL receptor cluster.

F–J) Cultures exposed to fl-Con A. F,G) Sparse culture of endothelial cells. Surface receptor sites for Con A are segretated into large patches and form perinuclear caps (F, G). H) Sparse endothelial culture fixed with formaldehyde (3.7%, 15 min, RT) before exposure to fl-Con A. A diffused fluorescence staining pattern over the entire cell surface area. I) A highly confluent and contact-inhibited monolayer of endothelial cells. A mostly peripheral, slightly aggregated, and random distribution of Con A receptor sites. i) Cells that contract during incubation and washing. J) Sparse culture of smooth muscle cells. Cells display a random, slightly clustered receptor distribution.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Single cells in suspension. The higher degree of receptor lateral mobility in sparse, as opposed to confluent, endothelial cell monolayers or to either sparse or confluent smooth muscle cell cultures was best demonstrated in suspension, after the cells had been released from the restriction imposed by their attachment to the substratum. Sparse endothelial cell cultures that were first incubated with LDL at 37°C and then exposed to fl-anti LDL at 4°C showed a high percentage (up to 70%) of cells with LDL capping when dissociated from their substrate with EDTA (Fig. 10A). Some of the cells showed minicaps or large aggregates similar to those observed with cells attached to a substratum (Fig. 10B). Small clusters randomly distributed over the entire cell surface area and no cap formation were observed in EDTA-dissociated, sparse endothelial cells that were dissociated with EDTA, fixed to prevent the internalization process, and then incubated in suspension with LDL and fl-anti LDL (Fig. 10C). A short (2–3 min) incubation at 37°C is required to dissociate the cell monolayers with EDTA into a single cell suspension. The possibility that the LDL capping observed in sparse endothelial cells could have been induced during the incubation with EDTA by a receptor cross-linking via the polyvalent anti-LDL molecules rather than by the initial incubation with the LDL particles themselves was tested by using fl-Anti LDL Fab fragments. These are monovalent and therefore incapable of receptor cross-linking and cap formation. Sparse endothelial cells that were exposed to LDL followed by incubation with either intact IgG anti-LDL or Fab fragment anti-LDL showed, after EDTA-dissociation, a similar percentage of cells with LDL capping, indicating that the LDL surface receptor sites were, in fact, redistributed in response to the initial incubation of the cells with LDL. In contrast to the results obtained with actively growing cells, cells from confluent endothelial monolayers that were similarly incubated with LDL and fl-Anti LDL showed, even in a single cell suspension, a clustered distribution of fluorescence but no caps (Fig. 10D). Suspensions of smooth muscle cells taken from either sparse or confluent cultures showed,

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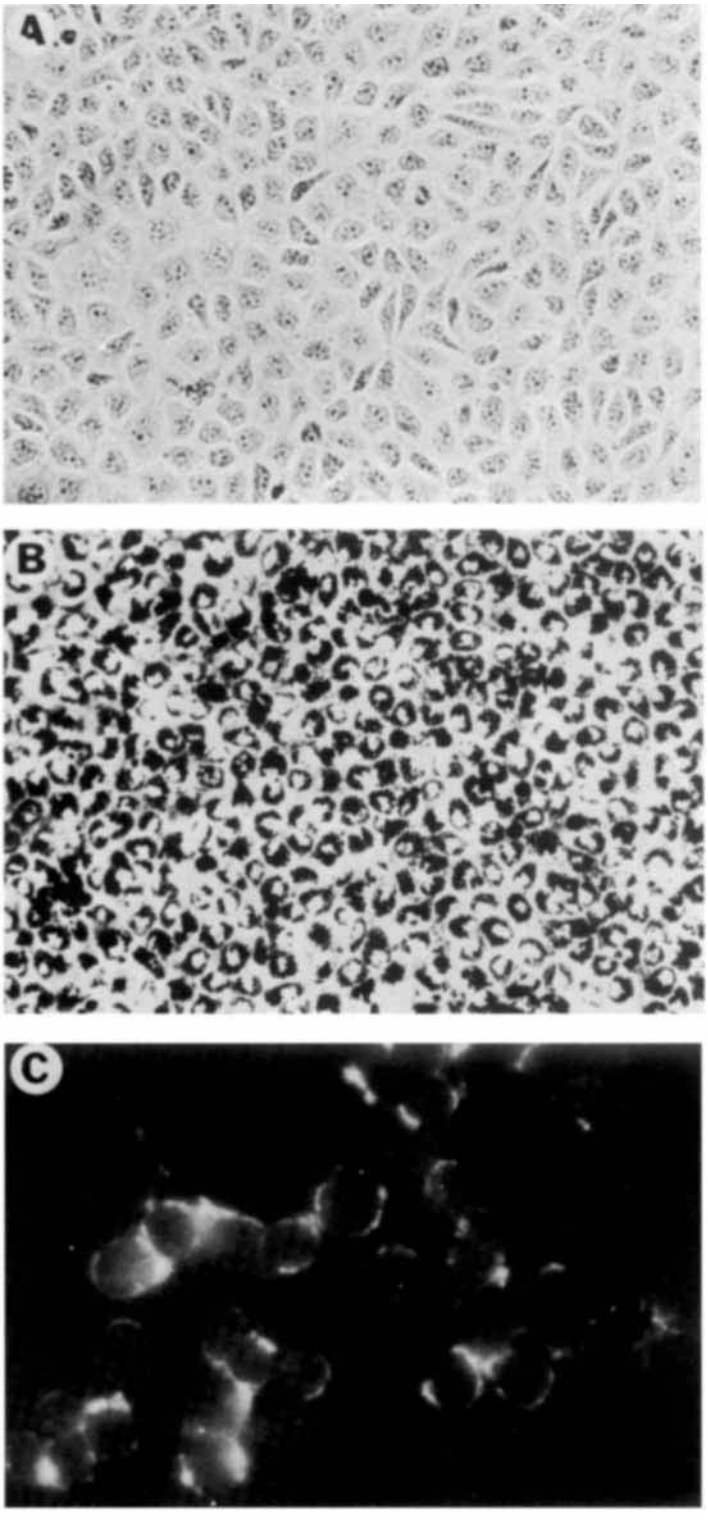
Fig. 10. Cell surface distribution of LDL and Con A receptor sites in EDTA-dissociated sparse and confluent vascular endothelial and smooth muscle cells.

A–E) Labeling with fl-Anti LDL. Cells attached to the tissue culture dish were incubated with LDL and fl-Anti LDL as described, dissociated with 0.03% EDTA solution, and spun; single cells in suspension were observed for their fluorescence staining pattern as described. A,B) represent cells from sparse endothelial cultures showing segregation of the LDL receptor sites into caps (A) or minicaps (B) in one pole of the cells. C) Sparse endothelial cells that were first dissociated with EDTA, fixed (3.7% formaldehyde, 15 min), and then incubated in suspension with LDL and fl-Anti LDL. A clustered but random distribution of fluorescence. D) Cells from a highly confluent and contact-inhibited monolayer of endothelial cells (the LDL receptor cites are randomly distributed in clusters over the entire cell surface area), focused on top (D) and at the cell periphery (d). E) Cells from a sparse culture of smooth muscle cells gave a highly aggregated but randomly distributed staining pattern.

F–J) Labeling with fl-Con A. EDTA-dissociated cells derived from sparse and confluent endothelial monolayers and from sparse cultures of smooth muscle cells were incubated with either fl-Con A or with native Con A followed by fl-anti-Con A, washed, and scored (single cells) for their fluorescence pattern as described. F,G) Sparse endothelial cells. In 70–80% of the cells the Con A receptor sites are segregated into large aggregates (G) or caps (F) at one pole of the cells. A similar percentage of Con A capping was obtained by using fl-anti-Con A rather than fl-Con A. H) Sparse endothelial cells that were fixed before exposure to fl-Con A. Cap formation by Con A is fully inhibited. I) Cells from a confluent endothelial monolayer incubated in suspension with Con A followed by fixation and incubation with fl-anti-Con A. A peripheral, ring-like distribution of Con A surface receptor sites. A slightly clustered distribution but no capping was obtained by a direct incubation with fl-Con A. J) Cells from a sparse culture of smooth muscle cells incubated as in (I). Receptors are slightly segregated and show no cap formation.



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under the same conditions, an aggregated staining pattern distributed over the entire cell surface, and there was no induction of receptor capping (Fig. 10E).

Experiments with Con A- and EDTA-dissociated cells gave results similar to those obtained with fl-Anti LDL. Cap formation by Con A was induced in 60–80% of the cells from sparse, actively growing endothelial cell cultures (Fig. 10F, G), whereas cells from a confluent monolayer showed a uniform ring-like distribution of fl-Con A (Fig. 10I). Cap formation by Con A was prevented by formaldehyde fixation before coating with fl-Con A (Fig. 10H). Patching, but not capping, of Con A receptor sites was observed in EDTA-dissociated smooth muscle cells (Fig. 10J). Similar results (capping with sparse endothelial cells and a ring-like distribution at confluence) were obtained with cells that were incubated directly with fl-Con A or first with Con A and then fixed and incubated with fl-anti-Con A in order that only Con A molecules on the cell surface and not those which might have been taken into the cells would be detected. These results indicate that the formation of a confluent endothelial cell monolayer composed of closely apposed and nonoverlapping cells is associated with a restriction of the lateral mobility of both LDL and Con A cell surface receptor sites [40].

Interaction of endothelial cells with cationized LDL. Experiments with cultured human fibroblasts and smooth muscle cells [22, 33] have indicated that the highly regulated process of LDL uptake can be bypassed by incubating the cells with cationized LDL. These positively charged LDL molecules bind to nonspecific sites on the cell surface from which they are taken up and degraded through a mechanism that does not involve the physiologic LDL receptor sites. Unlike the receptor-mediated binding and internalization of native LDL, the uptake and lysosomal degradation of cationized LDL was not affected by the density and organization of the endothelial cell cultures (Table I). Thus, staining of confluent endothelial cell monolayers with Oil Red 0 revealed substantial intracellular accumulation of lipid droplets after exposure to cationized LDL (Fig. 11B), but not after incubation with native LDL (Fig. 11A). The confluent cells also showed an active lysosomal degradation of ^{125}I-cationized LDL (Table I). As shown in Fig. 11C, a ring-like distribution of fluorescence and little or no receptor capping was observed with confluent endothelial cell monolayers that were incubated with cationized LDL followed by fl-Anti LDL and EDTA-dissociation into a single cell suspension. Therefore, the uptake of cationized LDL by these cells did not correlate with, nor did it induce, capping of the appropriate nonspecific, negatively charged surface receptor sites.

Our results therefore demonstrate that in sparse endothelial cells which internalize LDL the LDL receptor sites can be segregated into large patches or caps. Similar results were obtained when the lectin Con A, rather than LDL, was used as a probe. In contrast, confluent endothelial cell monolayers that can no longer internalize the bound lipoprotein

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Fig. 11. Oil Red 0 and fl-Anti LDL staining of confluent endothelial cells exposed to cationized LDL. A,B) Confluent monolayers of bovine aortic endothelial cells were exposed (48 h, 37°C) to either native LDL (600 μg/ml) or cationized LDL (15 μg/ml). The cell monolayers were then washed extensively, fixed, and stained with Oil Red 0 and hematoxylin. A) Cell exposed to native LDL. No accumulation of stained lipid droplets. B) Cells exposed to cationized LDL. Large numbers of Oil Red 0-positive inclusions. C) Surface distribution of the cell-bound cationized LDL molecules. Confluent endothelial monolayers were incubated (4 h, 37°C) with cationized LDL (10 μg/ml) and at 4°C with fl-Anti LDL and the fluorescence staining pattern determined after dissociation with EDTA as described under Materials and Methods. The cells display a ring-like distribution of fluorescence.

show no LDL- or Con A-induced receptor redistribution either before or after being released from their attachment to the substrate. These cells revealed at both 4°C and 37°C a clustered distribution of the LDL receptor sites, thereby showing a lack, or a very restricted type, of LDL receptor lateral mobility. A similar aggregated staining pattern was obtained with prefixed, either sparse or confluent, endothelial cells, indicating that surface receptors for LDL are located, even before the addition of LDL, in small clusters which only under sparse conditions and upon the binding of LDL can further move to form larger aggregates (cells attached to a substrate) or caps (cells in suspension) [40]. In contrast, if, instead of native LDL one uses cationized LDL, which binds nonspecifically to the cell surface, this positively charged ligand will be taken into the cells without a redistribution of its binding sites. Internalization of cationized LDL can therefore take place in both sparse and confluent cultures, regardless of the restriction on surface receptor mobility imposed upon vascular endothelial cells when they become highly organized and form a closely apposed cell monolayer.

Changes in the Cell Surface Occurring When Endothelial Cells Are Maintained in the Absence of FGF: FGF and the Normal Differentiation of the Vascular Endothelium

The preceding results demonstrated that the formation of a confluent endothelial cell monolayer is associated with structural (fibronectin, CSP-60) and function-related (receptor lateral mobility, adsorptive endocytosis) alterations in the cell surface. These changes can be looked upon as differentiation events which enable the endothelial cells to adopt in culture the morphologic appearance and metabolic behavior which characterize the endothelium of the large vessels. Of paramount importance to us was to determine the role of FGF in this expression of differentiated properties, ie, whether the presence of FGF during the phase of active cell growth is required for the normal differentiation of the endothelium [41]. For this purpose, cells were cultured in the absence of FGF and tested for growth behavior and surface-associated properties both at a sparse and confluent cell density.

Growth properties and morphologic appearance. Endothelial cells maintained without FGF had a much longer doubling time (60–78 h) than cultures maintained in the presence of FGF (18 h; Fig. 12A) and failed to proliferate when seeded at a high split ratio (Fig. 12B). They also adopt a strikingly different morphology (Fig. 13). The alterations in growth

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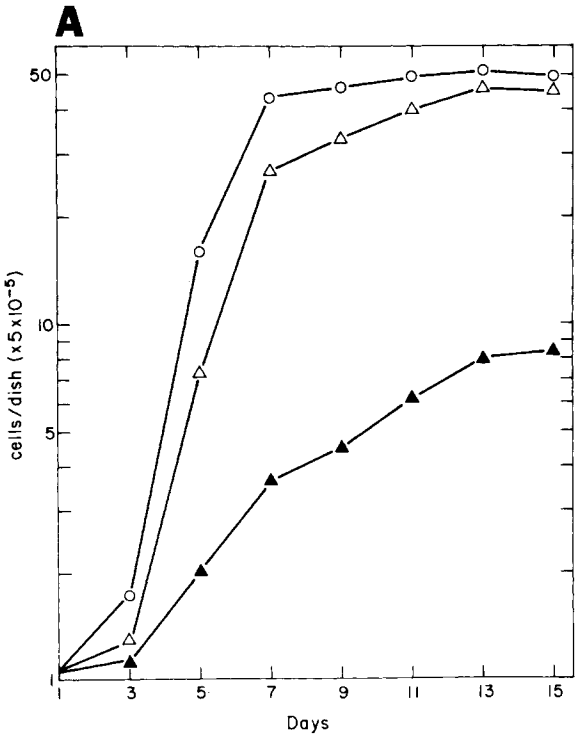
Fig. 12. Growth rate of vascular endothelial cells in the presence and absence of FGF. A) Vascular endothelial cells derived from the adult aortic arch (32 passages; 160 generations) were plated onto 35-mm dishes (2×10^4 cells per dish) in DMEM (H-16) supplemented with 10% calf serum. Duplicate cultures were counted every other day and the medium replaced every four days. ▲, Cells after three passages in the absence of FGF, no FGF was added. △, Cells after three passages in the absence of FGF; FGF was added on the third day after seeding and every other day thereafter. These cells adopted at confluence a perfect monolayer configuration indistinguishable from that of cells that were never subjected to FGF withdrawal. ○, Cells derived from endothelial cultures that were continuously maintained with FGF. FGF was added every other day. B) Confluent endothelial cell cultures maintained with (16 passages) (▲) or without (4 passages). (●) FGF were split at various ratios and cultured in the presence and absence of FGF (added every other day), respectively. The medium (DMEM + 10% calf serum) was replaced after four days and triplicate dishes were counted every other day. The number of cells after 9 days in culture is plotted as a function of the split ratio. The seeding level at a split ratio of 1:2 was 1.65×10^5 and 1.4×10^5 cells per 35-mm dish for cells maintained with and without FGF, respectively.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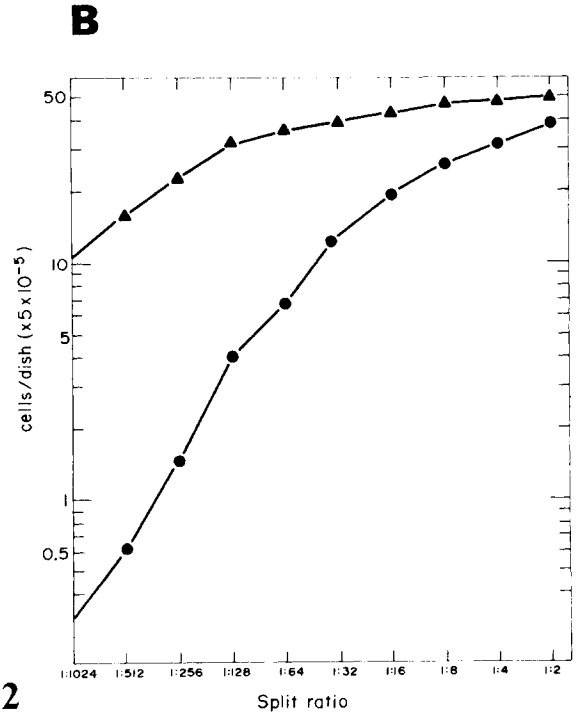
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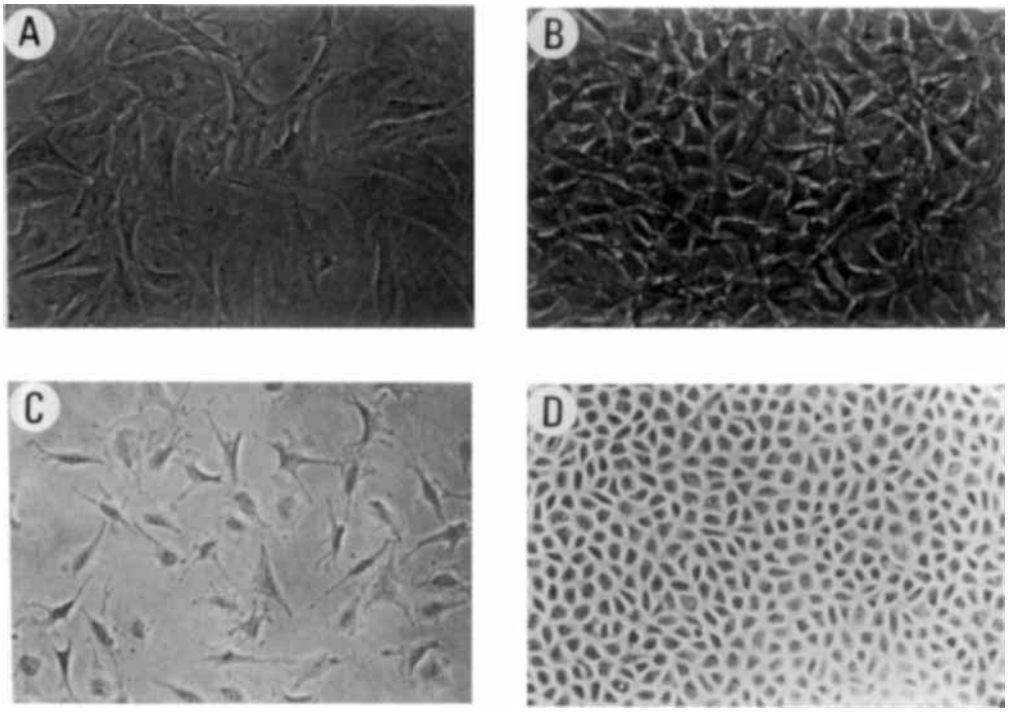


Fig. 13. Morphologic appearance of sparse and confluent vascular endothelial cultures maintained with and without FGF (phase contrast, × 150). A) Sparse endothelial cells maintained in the absence of FGF. The cells are 4–6 times larger and spread farther apart than sparse cells (C) seeded and maintained in the presence of FGF. B) Confluent endothelial cells after four passages (12 generations) in the absence of FGF. The cells grow on top of each other and in various directions. D) A confluent endothelial monolayer formed by cells (100 generations) maintained in the presence of FGF. The cells are highly flattened, closely apposed, and nonoverlapping.

behavior and morphologic appearance were best demonstrated after 3–4 passages (15–20 generations) in the absence of FGF. The cells, by then 4–6 times larger, failed to adopt a nonoverlapping monolayer configuration even after being split at a 1:4 ratio. Instead, at sparse density they were flattened and highly spread (Fig. 13A) and at confluence they grew on top of each other, leaving intercellular spaces (Fig. 13B). These cells exhibited a short lifespan, as reflected by vacuolization and cell degeneration after 30 generations in the absence of FGF. Readdition of FGF at an early passage to sparse or subconfluent cultures, or reseeding the cells at a low density but in the presence of FGF (Fig. 12A), resulted within 2–3 days in a resumption of cell growth and within 5–7 days in a morphologic appearance similar to that observed with endothelial cultures that are continuously maintained with FGF.

Fibronectin. As already described (Fig. 1C), the formation of a closely apposed endothelial cell monolayer is associated with a change in the localization of fibronectin so that it is no longer found on the apical cell surfaces but rather becomes a major component of the extracellular matrix and closely associated with the basal cell surface. In contrast, cells that were cultured in the absence of FGF showed a fibrillar distribution of

fibronectin over their apical cell surfaces, both when sparse (Fig. 14B) and even prior to the formation of cell-cell contacts (Fig. 14A) as well as late at confluence (Fig. 14C). Addition of FGF to sparse or subconfluent cultures previously deprived of FGF induced both cell proliferation and reorganization. This was associated with a change in the distribution of fibronectin which was much less or no longer detectable on the apical surface of cells that adopted the flattened, closely apposed, and nonoverlapping configuration typical of cultures maintained in the presence of FGF. In cells that were seeded in the presence of FGF, such a redistribution of fibronectin was revealed by the entire culture 5–7 days after reaching confluence, as previously observed with endothelial cells that were continuously maintained with FGF. When the FGF was added to a culture that had already reached subconfluence in the absence of FGF, the redistribution of fibronectin required a similar time interval (during which only 1–2 cell doublings took place), but was incomplete in those areas (up to 30% of the entire culture) which failed to adopt the appropriate morphology.

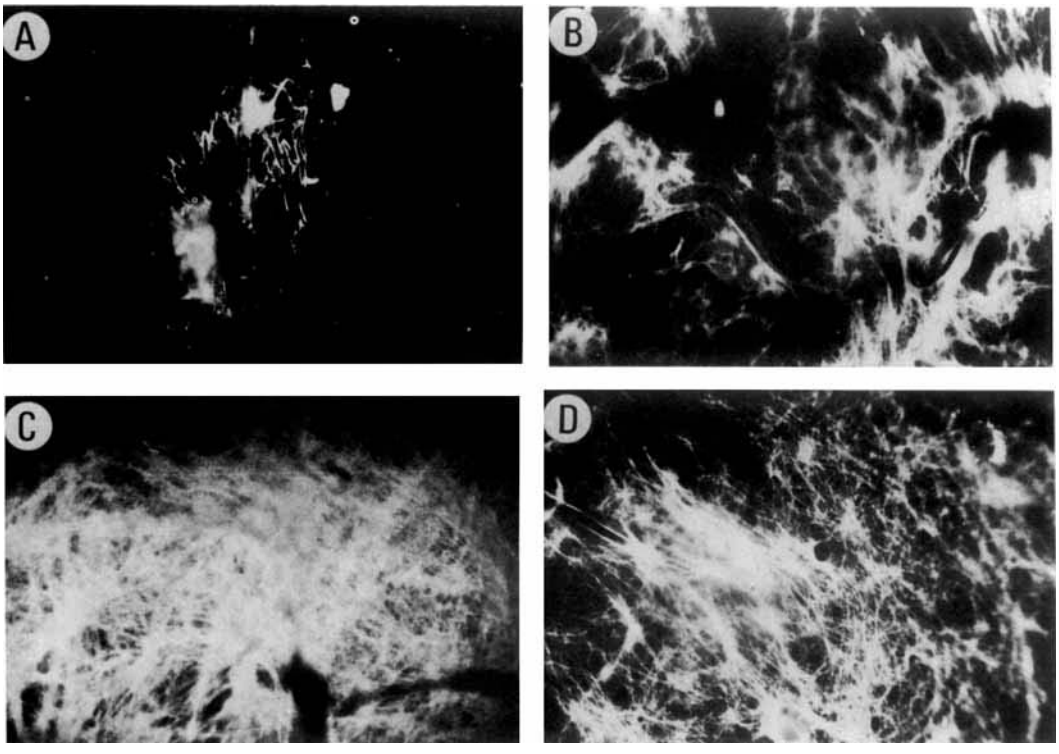


Fig. 14. Indirect immunofluorescence localization of fibronectin in endothelial cultures maintained without FGF. Vascular aortic endothelial cells maintained without FGF (three passages) were stained with rabbit anti-bovine plasma fibronectin and FITC goat anti-rabbit IgG as described in Materials and Methods. A) Sparse cultures prior to the formation of cell-cell contacts. Fibronectin is detected on top of the cells. B) Sparse and subconfluent cultures. Fibronectin is detected on top as well as in the areas of cell-cell contact C) Confluent cultures. Fibronectin is present in large quantities on top of the cells. D) Extracellular matrix left after removing the confluent cell layer with Triton X-100 (0.5%, 5 min, at 37°C).

Sparse and confluent cultures of endothelial cells maintained with or without FGF were subjected to the lactoperoxidase-catalyzed iodination in order to study further the differences in the surface localization of fibronectin and in particular in its quantity relative to other cell surface proteins. As demonstrated in Figure 15, cells that were cultured in the absence of FGF showed, even at a sparse density and even prior to the formation of cell-cell contacts (Fig. 15D, J), more fibronectin than that exposed for iodination in either sparse (Fig. 15A, G) or confluent (Fig. 15B, H) cultures that were maintained with FGF.

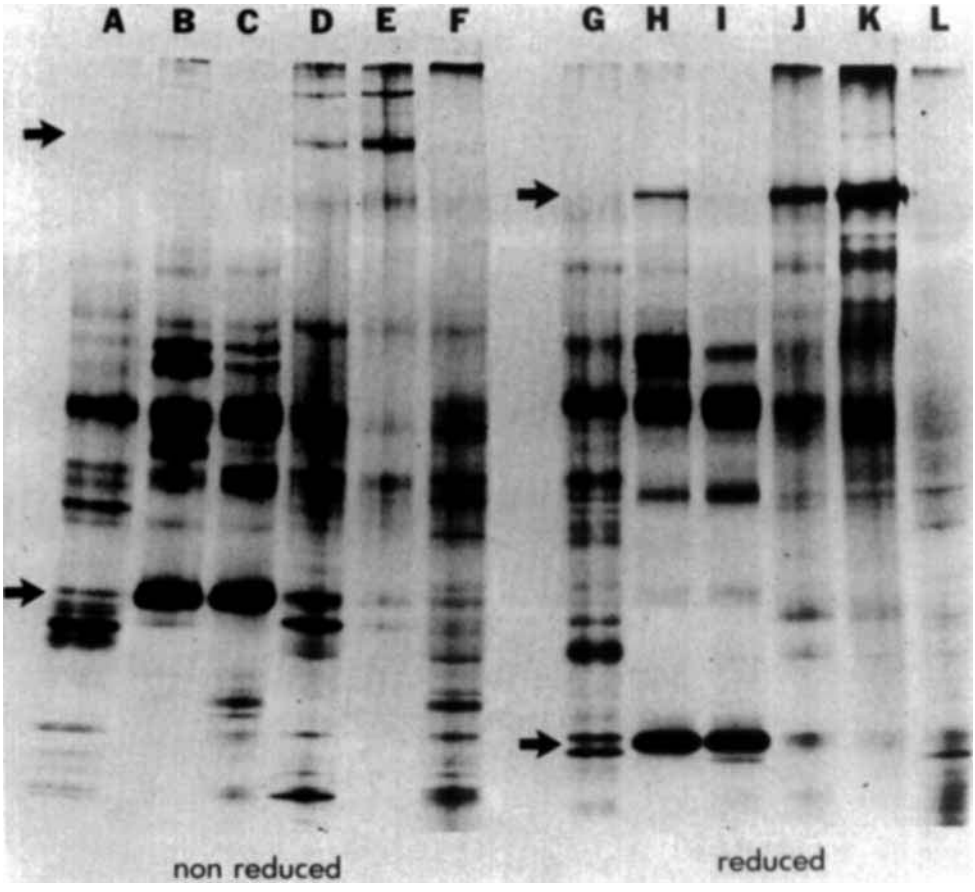


Fig. 15. SDS-polyacrylamide gel electrophoresis of lactoperoxidase-iodinated sparse and confluent endothelial cultures maintained with or without FGF. Washed cultures (maintained with or without FGF in a medium containing a fibronectin-depleted serum) were iodinated with ^{131}I and lactoperoxidase, 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,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,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, I) Confluent cultures exposed to a mild trypsinization (0.2 μg/ml, 45 min, 37°C). Fibronectin is largely removed, whereas CSP-60 is affected little or not at all. D,J) Sparse cultures prior to the formation of cell-cell contacts and maintained in the absence of FGF. Fibronectin is highly susceptible to iodination. E,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,L) Confluent cultures after a mild trypsinization. Fibronectin is largely removed. Arrows mark the positions of fibronectin and CSP-60.

In fact, in cells that were not exposed to FGF, fibronectin was the major component susceptible to iodination either at sparse density and to an even greater extent, late at confluence (Fig. 15E, K). When these cells were reseeded in the presence of FGF, they formed a confluent cell monolayer and showed, like cells that are continuously maintained with FGF, little or no fibronectin accessible to the lactoperoxidase-catalyzed iodination (Fig. 16D, I). In contrast, cultures that were reexposed to FGF after having already reached a high cell density showed only a partial removal of surface-associated fibronectin even after adopting a cell monolayer configuration (Fig. 16C, H). These experiments had also demonstrated that the fibronectin present on the cell surface is bonded by disulfides or otherwise (eg, transglutaminase) 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 reduction of the samples with DTT (Figs. 15 and 16).

Immunofluorescence staining of the extracellular material left after removal of the confluent cell layer with Triton X-100 had demonstrated a massive accumulation of fibronectin in the extracellular matrix produced by cells maintained with (Fig. 1D) or without (Fig. 14D) FGF. Endothelial cells that were cultured in the absence of FGF therefore resembled fibroblasts [28] or smooth muscle cells [29] in having fibronectin associated with both their upper and lower cell surfaces. The expression at confluence of fibronectin underneath the cell layer but not on the apical cell surface might therefore be a unique differentiated property of endothelial cells that are maintained in the presence of FGF and form a monolayer composed of flattened and nonoverlapping cells [41].

The production of fibronectin by sparse and confluent endothelial cultures maintained with or without FGF was further studied by exposing the cells to [^{35}S] methionine and subjecting both the cell layer (Fig. 17K–N) and tissue culture medium (Fig. 17A–J) to SDS slab gel electrophoresis before and after immunoprecipitation with antifibronectin antiserum. As shown in Table II, antifibronectin precipitated less than 3% of the total [^{35}S]-labeled proteins that were secreted into the medium of cells that were cultured with FGF. In contrast, 20–25% of the total [^{35}S]-labeled proteins were precipitated from the culture medium of cells that were maintained in the absence of FGF. When analyzed on SDS polyacrylamide gels (Fig. 17A, 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 times more fibronectin per cell than sparse and confluent cells cultured in the presence of FGF, respectively (Table II).

A higher production of fibronectin in the absence of FGF was also observed when cell extracts, rather than the tissue culture medium, were analyzed for the presence of [^{35}S]-labeled fibronectin (Fig. 17M, N). These results, together with the immunofluorescence and surface iodination experiments, indicate that the production and distribution of fibronectin are sensitive to changes in cell organization and growth behavior induced by FGF.

CSP-60. The presence on the cell surface of a specific protein (CSP-60) is a characteristic property of endothelial cells that form a highly confluent cell monolayer (Figs. 3–8). This protein cannot be detected by lactoperoxidase-catalyzed iodination in actively growing and unorganized endothelial cultures. Likewise, CSP-60 was no longer exposed for iodination in disorganized endothelial cell monolayers and was not present in sparse or confluent cultures of vascular smooth muscle cells that grow in multiple layers [32]. As demonstrated in Figure 15, CSP-60 was not detected in sparse cultures maintained with (lanes A and G) or without (lanes D and J) FGF but was largely exposed for iodination in confluent cultures

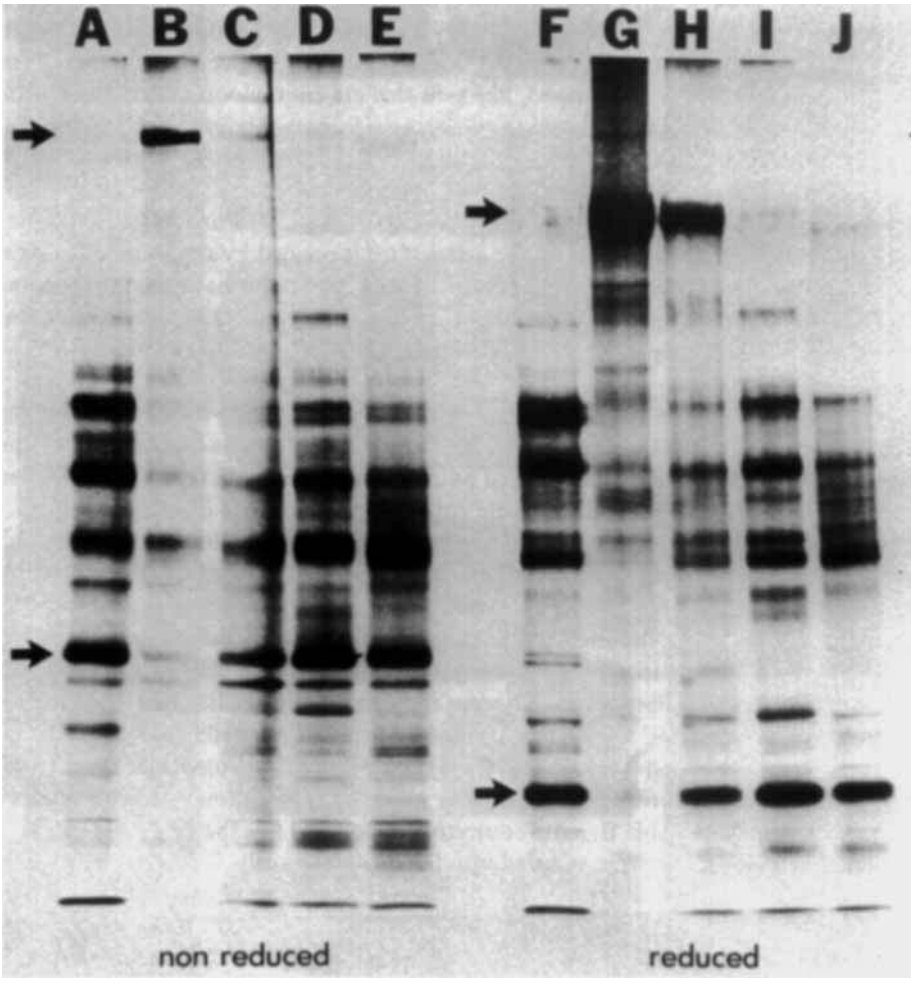


Fig. 16. Cell surface iodination pattern of overlapping and reorganized endothelial cells before and after being reexposed to FGF. Endothelial cells maintained for three passages in the absence of FGF were seeded at a split ratio of 1:8 and FGF was added (every other day) starting on day 2 or day 7 after seeding. Control cultures (not exposed to FGF) and reorganized cultures (reexposed to FGF) were iodinated at confluence (14 days after seeding) and analyzed by gradient (5–15%) polyacrylamide slab gel electrophoresis before (ianes A–E) and after (lanes F–J) reduction with 0.1 M DTT. A,F) A confluent monolayer of endothelial cells that were continuously maintained in the presence of FGF. CSP-60 appears as a major band. B,G) Confluent but unorganized endothelial culture maintained for four passages without FGF. Fibronectin appears as a major band; CSP-60 is missing. C,H) Same cells as in (B) and (G) exposed to FGF at a subconfluent density (7 days after seeding) and labeled 7 days later. Most of the cultures adopt a monolayer configuration, although some unorganized areas (about 20% of the culture) are still present. Both fibronectin and CSP-60 are exposed for iodination. D,I) The same cells as in (B) and (G) exposed to FGF at a sparse density (3 days after being split at a 1:8 ratio) and labeled 12 days afterwards, when the cells were highly organized and closely apposed. CSP-60 appears as a major band, whereas fibronectin is detected in small amounts as in cells that are maintained continuously with FGF. E, J) – FGF culture exposed when subconfluent to a medium conditioned by a confluent monolayer of endothelial cells and iodinated 7 days afterwards. Arrows mark the positions of fibronectin and CSP-60.

TABLE II. Secretion of Fibronectin Into the Growth Medium of Endothelial Cells Maintained With or Without FGF

Cells	Total [^{35}S]-labeled proteins[a] (cpm × 10^{-6} per 10^6 cells)	[^{35}S]-labeled material precipitated with antifibronectin[b] (cpm × 10^{-6} per 10^6 cells)	% Fibronectin in total [^{35}S]-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 and exposed to [^{35}S] methionine (65 μCi/ml, 20 h) in DMEM containing 10 μM methionine and 0.5% bovine serum as described in the legend to Figure 17. Aliquots (10 μl) of the growth medium were taken for precipitation with 10% boiling TCA and the entire 1 ml 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 in Materials and Methods. The same amounts of fibronectin were precipitated by using either 5 μl or 50 μl of each of these antisera preparations.

[a] Sparse and confluent endothelial cells maintained in the presence of FGF contained 320 and 260 μg protein per 10^6 cells, whereas sparse and confluent cells maintained in absence of FGF contained 1,810 and 1,462 μg protein per 10^6 cells, respectively.

[b] Nonspecific precipitation with nonimmune rabbit serum was carried out under each condition and the values were substracted from those obtained with antifibronectin. Nonspecific precipitation did not exceed 1% of the total [^{35}S]-labeled proteins secreted into the medium.

that were maintained in the presence of FGF and had adopted a closely apposed cell monolayer configuration (Fig. 15B, H). In contrast, endothelial cells that were cultured and reached confluence in the absence of FGF showed little or no CSP-60 even at late confluence (Fig. 15E, K). Readdition of FGF to sparse or subconfluent endothelial cultures that were maintained without FGF was associated with a reappearance/exposure of CSP-60 concomitant with the adoption of a closely apposed cell monolayer configuration (Fig. 16C, D and H, I). The presence of CSP-60 on the cell surface was not affected by a mild trypsinization (Fig. 15C, I) which did not disrupt the cell layer. Since under the same conditions fibronectin was largely or completely removed from the cell surface, it is unlikely that CSP-60 is not detected in cells maintained without FGF because of being masked by the meshwork of fibronectin which, under these conditions, is found on top of the cells.

Thrombogenic properties. Like the vascular endothelium in vivo [42], sparse and confluent (Fig. 18A) endothelial cells maintained with FGF have an apical, nonthrombogenic surface to which platelets cannot adhere. Thus, no platelets, or less than one platelet per cell, were found on top of cells that were incubated (30 min, 37°C) with human platelets (2×10^8 platelets/m) and washed extensively (Fig. 18B). In contrast, when endothelial cells maintained without FGF (Fig. 18C) were tested for the same property, they exhibited a high platelet-binding capacity (20–50 platelets per cell) both at a sparse and confluent (Fig. 18D) cell density. As shown in Fig. 18D, the platelets were bound singly to the apical surface of these cells and no aggregation of the attached platelets took

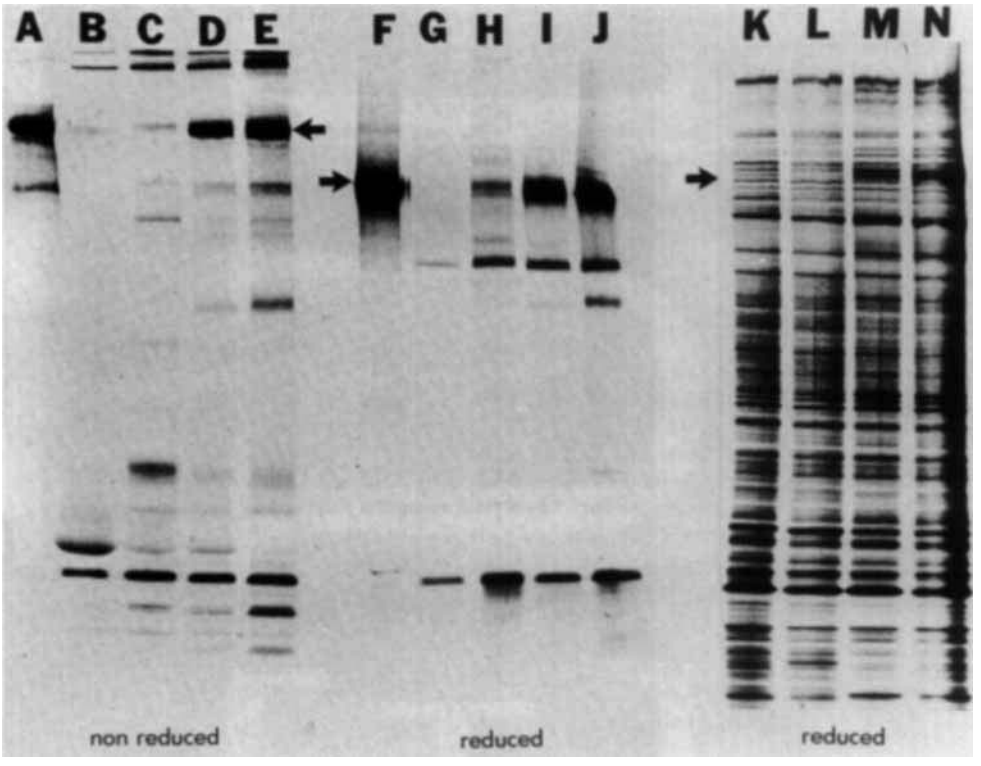
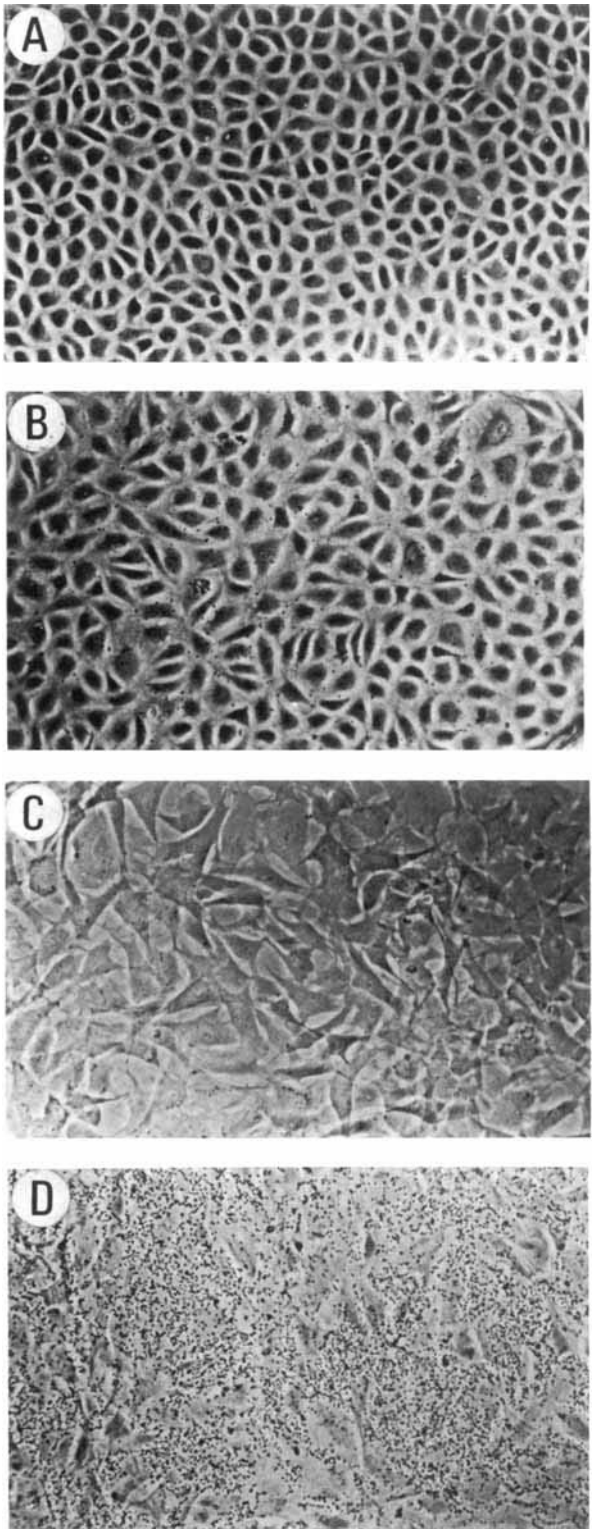


Fig. 17. Electrophoresis of [^{35}S] methionine-labeled proteins present in cell extracts and tissue culture medium of sparse and confluent endothelial cultures maintained with or without FGF. Sparse (actively growing) and confluent (10 days after reaching confluence) endothelial cultures maintained with or without (three passages) FGF were exposed to [^{35}S] methionine (65 μCi/ml, 20 h) in DMEM containing 10 μM methionine and 0.5% bovine serum. Cell extracts (in sample buffer) (lanes K–N) and 5–15 μl aliquots of the medium (lanes A–J) containing 50,000 protein-bound (TCA-precipitable) cpm were then subjected to a gradient (5–16%) polyacrylamide slab gel electrophoresis either before (lanes A–F) or after (lanes F–N) reduction with 0.1 M DTT. A,F) Immunoprecipitation pattern (antifibronectin followed by anti-IgG) of growth medium taken from sparse endothelial cells cultured in the absence of FGF. B, G) Proteins secreted into the medium by sparse cells maintained with FGF. C,H) Proteins secreted by sparse cells cultured in the absence of FGF. D,I) [^{35}S]-labeled proteins secreted into the medium by confluent cells that were maintained with FGF. E,J) Medium of confluent cells cultured in the absence of FGF.K–N) Cell extracts of sparse (K,M) and confluent (L,N) endothelial cultures maintained with (K,L) or without (M,N) FGF. Arrows mark the positions of fibronectin dimers (460K, nonreduced samples) and monomers (230K, reduced samples).

Fig. 18. Adherence of platelets to confluent vascular endothelial cultures maintained with or without FGF. Confluent endothelial cultures maintained in the absence (three passages) or presence (during the phase of logarithmic growth) of FGF were incubated with human platelets (2×10^8/ml, 30 min, 37°C), washed, and observed by phase microscopy (× 150) as described under Materials and Methods. A,B) Cells maintained with FGF. Very little or no platelets can be seen (B) attached to the upper surface of cells that adopt a monolayer configuration as in (A). In contrast, most of the upper surface of unorganized cultures (C) maintained in the absence of FGF is covered with platelets which attached singly and do not form aggregates (D).


A
B
C
D

place. In order 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 ^{14}C-serotonin. The reaction medium containing unattached platelets and any serotonin which might have been released during binding were then removed and the platelets collected by centrifugation. The cell layer was then extensively washed and solubilized in 0.1 N NaOH. The cells, platelets, and platelet-free supernatant were counted separately to determine the distribution of ^{14}C-serotonin. As shown in Table III, 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 ^{14}C-serotonin content (Table III). When the radioactivity associated with the cell layer was measured, it was found, as already observed (Fig. 18), that endothelial cells maintained without FGF bind platelets to an extent that is 9 and 25 times higher (when calculated per culture dish and per cell, respectively) than cells that were cultured in the absence 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 lactoperoxidase-catalyzed iodination) (Fig. 15F, L), decreased the adherence of platelets by no more than 40%. Cultures maintained in the absence of FGF readopted within 8–10 days after being exposed to FGF the configuration of a highly flattened monolayer composed of closely apposed and nonoverlapping cells. These cells, like cells that were continuously maintained with FGF, exhibited a nonthrombogenic apical surface to which platelets did not adhere.

Barrier function. Vascular endothelial cells, by virtue of their location at the inner surface of blood vessels, are exposed to various substances at concentrations and proportions far different from those found in the extravascular region and are therefore expected to possess unique properties, both as a barrier and as a transport system. Such properties

TABLE III. Binding of Platelets to Vascular Endothelial Cells Maintained With or Without FGF

Cells	Cell-bound platelets (^{14}C-serotonin)		Released ^{14}C-serotonin
	cpm/dish	cpm/10^6 cells	(cpm/dish)
+ FGF cultures	1,192	1,748	5,930
– FGF cultures	9,504	50,553	6,097
– FGF cultures pretreated with trypsin[a]	5,227	27,803	5,944
No cells	–	–	6,311
No cells + thrombin (1 unit/ml)	–	–	87,653

Platelets (2×10^8) prelabeled with ^{14}C-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 unit/ml) induced a 90% release of the ^{14}C-serotonin that was introduced into the platelets.

[a]Worthington twice-crystallized trypsin (0.5 μg/ml, 2 h, 37°C in DMEM).

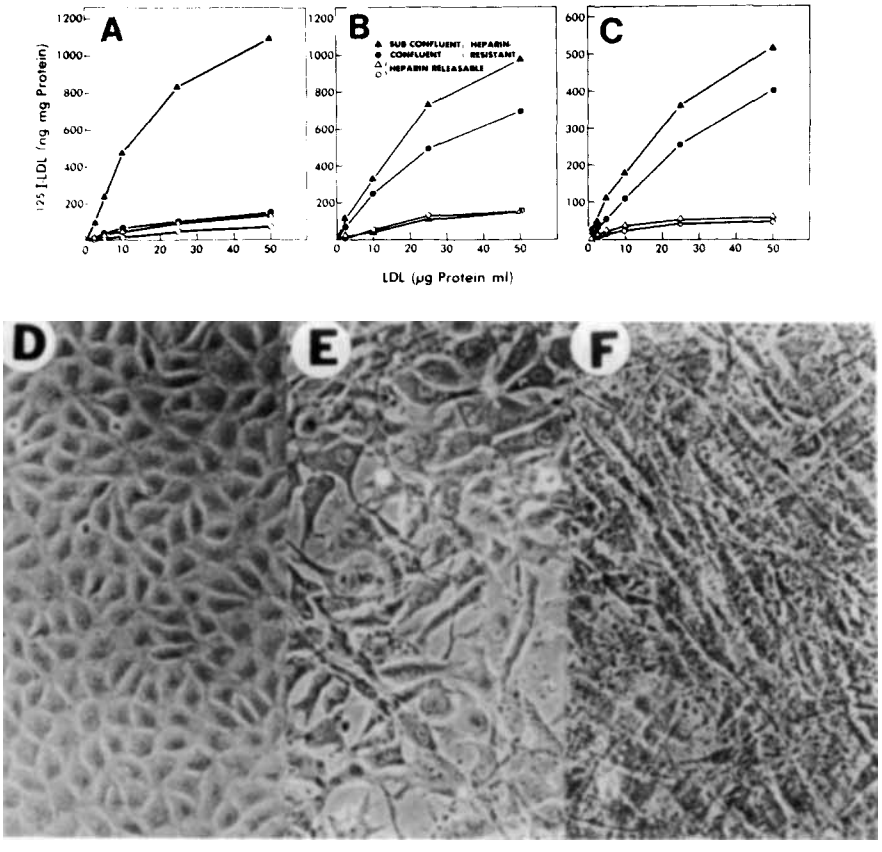


Fig. 19. Binding and uptake of ^{125}I-LDL by sparse and confluent cultures of endothelial and smooth muscle cells. A–C) LDL binding and internalization. A) Adult bovine aortic endothelial cells. B) Adult bovine aortic endothelial cells maintained in the absence of FGF for four passages. C) Adult bovine aortic smooth muscle cells. D–F) Morphologic appearance of the confluent cultures described under (A), (B), and (C) at the time of the experiment, respectively. The endothelial and smooth muscle cells were exposed to LDL before (▲, △) and 8 days after (●, ○) reaching confluence. Cells were seeded, maintained, and tested for specific binding (△ and ○, accessible for heparin release) and internalization (▲ and ●, heparin-resistant) of ^{125}I-LDL, as described under Materials and Methods. The results demonstrate that LDL internalization by different cell types can be correlated with their morphologic appearance at a high density.

are of particular significance in preventing an excess of plasma lipoproteins from accumulating in the subendothelial regions and in the smooth muscle cells of the arterial wall. In previous studies, we have demonstrated that when endothelial cells form a confluent cell monolayer, although the LDL still binds to cell surface receptors, the molecules are no longer internalized and, hence, there is no degradation of the lipoprotein [24–26]. Since morphologic as well as surface changes are induced by maintaining the endothelial cells without FGF, we have studied whether these alterations can in turn be expressed by changes in the permeability barrier function of these endothelial cells. For this purpose, sparse and confluent endothelial cultures maintained with or without FGF were tested for

their ability to bind and internalize ^{125}I-labeled LDL particles. As shown in Figure 19, endothelial cultures maintained without FGF (Fig. 19B) can, like vascular smooth muscle cells (Fig. 19C), internalize the receptor-bound LDL particles both at a sparse and confluent cell density. These cells can grow on top of each other and form multiple cell layers at confluence (Fig. 19E, F). In contrast, vascular endothelial cells that are maintained in the presence of FGF adopt at confluence a monolayer configuration (Fig. 19D) and as a result can bind but no longer internalize the LDL particles (Fig. 19A). As demonstrated in Figure 19, sparse endothelial cultures exhibited a similar degree of LDL binding and internalization when maintained either with or without FGF. The difference between the two types of cultures was expressed only late at confluence and was reflected by the lack of LDL uptake in cells that were maintained with FGF. Since the cell surface area becomes smaller once a tightly packed monolayer is formed, cells in a confluent endothelial monolayer showed up to a twofold to threefold decrease in LDL-binding capacity compared with sparse (+) FGF cultures or with either sparse or confluent cultures that were maintained without FGF.

DISCUSSION

The present study summarizes various observations on the role of the cell surface in determining the normal morphologic appearance and physiologic function of the vascular endothelium. These cells exhibit unique adhesive interactions (both in terms of cell-cell interactions and adhesion to the basal lamina) as well as a nonthrombogenic apical surface to which platelets cannot adhere. These apparently contradictory properties imply that a certain degree of membrane asymmetry has to be achieved as the endothelial cells differentiate to form a monolayer composed of nonoverlapping cells that no longer divide. The adoption of such a morphology is in turn essential in order that the endothelium function both as a nonthrombogenic surface and as a regulator of the system's permeability to plasma constituents. By using FGF and cultured endothelial cells that mimic perfectly their in vivo counterparts, we have demonstrated that a cell surface asymmetry is in fact acquired late at confluence and concomitant with the formation of a highly organized cell monolayer. In terms of structural rearrangements, this was reflected by disappearance of fibronectin from the apical cell surface; appearance of a specific cell surface protein (CSP-60) not detected in actively growing cells; production of a highly thrombogenic basal lamina composed mostly of a fibronectin-collagen-proteoglycan meshwork; and restriction of the lateral mobility of various cell surface receptor sites. In terms of physiologic function, the confluent endothelial cell monolayer fulfills the requirements of having a nonthrombogenic apical surface and serving as an efficient barrier against the uptake of LDL, which is the main cholesterol carrier in blood. Both the structural and functional attributes exhibited by a confluent endothelial cell monolayer can be regarded as differentiated properties and they have been shown to depend on the presence of FGF during the active phase of cell growth [41].

Respective Roles of Fibronectin and CSP-60

The presence of large amounts of fibronectin in cells that grow on top of each other, and the rapid reorganization of trypsinized endothelial cells despite the removal of fibronectin, suggest that fibronectin is not an essential factor in the formation of highly organized cell monolayer. Fibronectin is a major constituent of the basal lamina produced at confluence by endothelial cells [1–3]. it is therefore likely that with these cells the primary function of fibronectin is to enforce cell-to-substrate adhesion rather than to be

involved in determining the final organization of the tissue. If one considers the turbulence, the pressure variation, and the speed of blood flow in the aortic arch, it becomes evident why the endothelial cells must develop a special means of remaining attached as a cell monolayer to their basal lamina. Such a means could be the production of great amounts of extracellular material, such as fibronectin, which primarily functions to enforce cell adhesion [43, 44]. It is therefore suggested that because of their morphology (monolayer), shape (flattened), and situation (inner layer of the arteries and veins), vascular endothelial cells are called upon to make a major contribution to the production of the fibronectin found in the basement membrane of the blood vessels as well as that found in the plasma, where it is present as CIG. In contrast, the appearance on the cell surface of CSP-60 was, under normal and various experimental conditions, always correlated with the formation of a confluent cell monolayer but not with substrate adhesion and flattening of the endothelial cells. This protein can therefore play a role in the interaction between cells which leads to the adoption of a monolayer configuration composed of closely apposed and nonoverlapping cells.

Receptor Restriction, Production of a Fibronectin Meshwork, and Formation of an Endothelial Barrier to Receptor-Mediated Uptake of LDL

Experiments with human fibroblasts have indicated that a lateral receptor mobility is not required for the receptor-mediated uptake of LDL. In these cells the LDL receptor sites are predominantly located in coated regions of the plasma membrane, which can invaginate to form coated vesicles [39]. It has been observed, however, that the internalization of various polypeptides (α_2-macroglobulin, insulin, epidermal growth factor) is preceded by patching, probably into coated pits, of specific receptor sites that are otherwise diffusely distributed over the entire cell surface area [37]. The present results on the inhibition in confluent endothelial cultures of both the uptake of LDL and the lateral mobility of the appropriate cell surface receptor sites suggest that receptor redistribution might also be involved in the internalization of LDL by actively growing vascular endothelial cells. It therefore seems that a requirement for a certain degree of ligand-receptor lateral mobility is the more general mechanism for internalization via adsorptive endocytosis, although various cell types and various receptor sites on a given cell type might require a very limited degree of such receptor distribution or none at all. This has been demonstrated by showing that the internalization of native LDL by vascular smooth muscle cells and of cationized LDL by endothelial cells was not correlated with a detectable receptor redistribution.

Bornstein et al [45] have proposed a model in which an external fibronectin-collagen meshwork interacts, via transmembrane and cytoplasmic peripheral proteins, with microfilaments subjacent to the plasma membrane. This interaction might, among other effects, restrict the lateral mobility of various receptor sites in the membrane plane [45, 46]. Evidence of such an interaction was provided by these [45] and other investigators [47, 48]. There is also evidence of a restricting effect of the extracellular matrix, consisting mostly of fibronectin, on Con A receptor lateral mobility and cell agglutinability [49, 50]. Using indirect immunofluorescence and surface iodination techniques, we have demonstrated that late at confluence fibronectin becomes less exposed on the apical endothelial cell surface and is primarily produced as disulfide-bonded complexes toward the substrate underlying the cell layer. This is associated with a reorganization of the cellular microfibrillar system, which no longer shows any linkage with the apical cell surface but rather lies parallel to both the basal and apical cell surfaces [2]. It therefore seems possible that an "exoskeleton" of the matrix, consisting of fibronectin and perhaps other proteins or glyco-

proteins, might interact indirectly with cytoskeletal elements or be directly cross-linked to other components of the cell surface [16], thereby leading to a change in membrane dynamics and organization. This might affect not only the freedom of surface receptor lateral mobility but also the rigidity of the membrane in general, thus inhibiting processes such as invagination and formation of endocytotic vesicles. That this could, indeed, be the case is strongly supported by the experiments of Nicolau et al [51], which show a significant stiffening of the cell membrane at a saturation density of growth-controlled cells.

Contact inhibition was shown to inhibit phagocytosis [52] and toxin uptake [53] in cultured epithelial sheets and 3T3 fibroblasts, respectively. We have also observed a two-fold to threefold inhibition of the bulk phase pinocytosis (measured by the uptake of either ^{14}C-inulin or ^{14}C-sucrose) upon the formation of a confluent endothelial cell monolayer. Experiments with membrane vesicles isolated from rapidly growing and quiescent 3T3 cells have demonstrated that the inhibition of uridine transport at confluence is due to a modification in the structure of the cell membrane rather than in the overall cell metabolism [54]. In the case of the vascular endothelium, such a structural modification was reflected by the appearance of CSP-60 on the apical cell surface and the redistribution of fibronectin, which was no longer found on top of the cells but rather accumulated in the basal lamina. We have also observed an increased production of a 58,000 MW protein in highly organized and resting endothelial cultures versus sparse and actively growing cells (Fig. 20, protein No. 1). This difference is most significant in view of the greatly reduced synthesis of other cellular proteins (in terms of protein types and total incorporation of [^{35}S] methionine) that occurs late at confluence. Preliminary studies suggest that the 58,000 MW protein migrates in a manner similar to the 58K intermediate (10 nm) filament, which has been shown to be a major cytoplasmic structure of many eukaryotic cells. This protein might play an important role in maintaining the flattened and closely apposed morphology of confluent endothelial cells (Vlodavsky, Savion, and Gospodarowicz, manuscript in preparation).

FGF and the Normal Differentiation of Vascular Endothelial Cells

In previous studies we have emphasized the role of FGF as a potent mitogen for vascular endothelial cells and an essential factor in developing clonal endothelial cell populations. We have now compared endothelial cells maintained with and without FGF in order to study whether changes other than those related to cell proliferation can be induced by the presence of FGF [41]. 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 asymmetry. These properties reflect an advanced stage of differentiation in which the upper cell surface is nonthrombogenic 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 in order to elucidate the factors which allow the endothelium of the large vessels to form a nonthrombogenic surface, to resist high pressure and Sheer forces, and to function as a selective barrier against plasma components and particularly lipoproteins.

The present results have demonstrated that cells maintained in the absence of FGF exhibit, in addition to a much slower growth rate, morphologic 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 and nonoverlapping cells; b) a loss of cell surface asymmetry, as

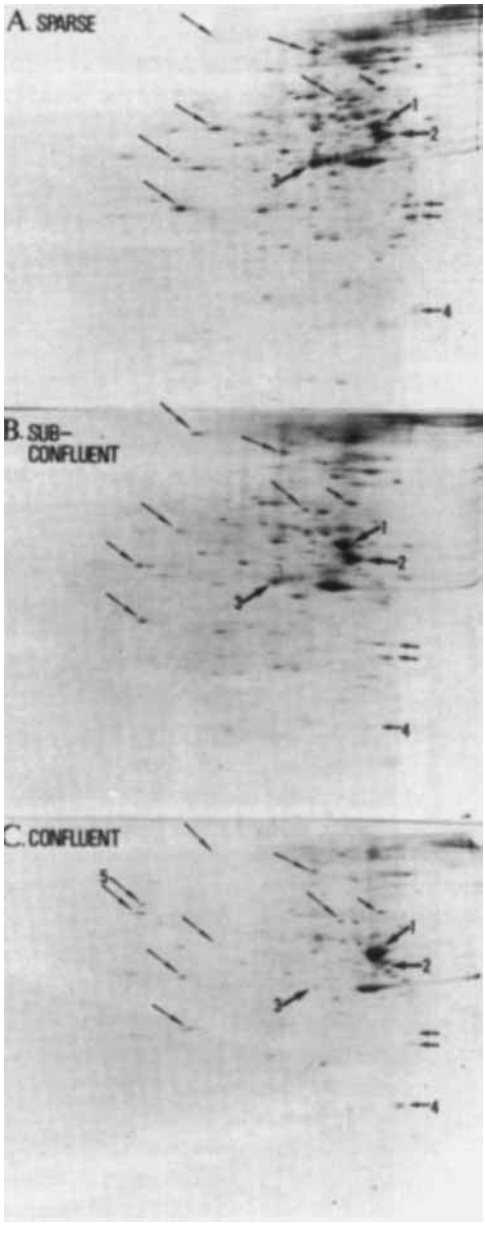


Fig. 20. Two-dimensional electrophoresis pattern of proteins synthesized by sparse, subconfluent, and confluent vascular endothelial cells. Endothelial cultures maintained with FGF were exposed to [^{35}S] methionine (200 μCi/ml, 60 min, 37°C), washed, and lysed with isoelectric focusing sample buffer (9.5 M urea, 2% NP-40, 2% ampholytes, pH 3.5–10,5% β-mercaptoethanol). Then 500,000 protein-bound cpm was subjected to two-dimensional gel electrophoresis (nonequilibrium, pH gradient electrophoresis followed by SDS gel electrophoresis on a 10–16% gradient polyacrylamide slab gel). A) Sparse, actively growing endothelial cells. B) Subconfluent cultures which are not yet organized into a cell monolayer. C) Confluent, highly organized, and resting monolayer of endothelial cells. Arrows mark the main differences between the three autoradiograms (exposure time was 4 days).

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 by both sparse and confluent cultures; d) a lack of CSP-60 (detected by the lactoperoxidase-catalyzed iodination technique), a cell surface protein susceptible to iodination only in cells that adopt a monolayer configuration; and e) a failure to form an effective barrier against low density lipoprotein. Exposure of the cultures to FGF induces a restoration of the normal endothelial characteristics concomitant with the adoption of a flattened cell monolayer morphology [41]. The physiologic significance of the alterations listed above is in particular demonstrated by the inability of the non-contact-inhibited (minus FGF) endothelial cells to protect the subendothelial layer against an overaccumulation of LDL cholesterol. This might initiate the formation 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 is quite new. Whether 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 prior to the formation of cell-cell contacts, which suggests, 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. It should also be emphasized in this regard 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 a normal growth rate, morphologic 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.

Preliminary results from this laboratory (Fig. 16E, J) indicate that a reversion of highly overlapping endothelial cells can also 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 [55]. Therefore, endothelial cells that are seeded at a high density (1:4 split ratio), unlike cells that are seeded at a low or clonal density, may, by conditioning the medium, adopt a cell monolayer configuration and the associated differentiated properties, even in the absence of an added factor such as FGF.

The present study presents new aspects of the pleiotropic interaction between a mitogen and a given cell type. This is best demonstrated by the observation that FGF can induce both 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 the survival as well as the differentiation, of neuronal cells.

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 survival and differentiating effects are observed at different times in the cells' lives. Our recent studies with rat pituitary cells have also demonstrated an epidermal growth factor (EGF) effect on gene expression and differentiated functions (synthesis of prolactin and growth hormone) which was not associated with a mitogenic response [56]. In contrast, with vascular endothelial cells FGF can simultaneously be both a proliferative and differentiating agent. Its long-term effects on the differentiation and physiologic 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 proliferative index.

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NOTE ADDED IN PROOF

In a recent communication [1], the group of Collaborative Research (CR) has described the identification of a new growth factor, endothelial cell growth factor (ECGF) isolated from brain tissue. They also report, in contrast to our own findings [2], that neither brain nor pituitary FGF had any activity on human umbilical endothelial cells. In this communication, the extraction procedure of pituitary and brain FGF used by CR is described for the first time. Although they claimed in the past that their purification was similar to the one we have described [3, 4], it differs from our own at a single but critical point. Although we insisted that brain and pituitary tissue be extracted at pH 4.5 in the presence of 0.15 M $(NH_4)_2 SO_4$, CR has been extracting the tissues in saline, ie without pH control and probably near neutrality (pH 6.5–7.0). In 1975 we reported the characterization from brain and pituitary tissue of a factor we named by its activity on the first cell type upon which it was tested, viz myoblast growth factor [5]. This factor is in all likelihood the same as the so-called ECGF and is preferentially, if not exclusively extracted at basic (8.5) as well as neutral (7.0) pHs, but not at acidic pHs [5]. In contrast, as already reported [5], FGF can only be extracted at an acidic pH of 4.5. Extraction at neutral or basic pHs results in the solubilization of little or *no* activity [5]. It is therefore not surprising that CR reports that, when a neutral pH of extraction was used, brain or pituitary FGF had no activity on endothelial cells, since this factor was never extracted from these tissues.

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