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Appearance in confluent vascular endothelial cell monolayers of a specific cell surface protein (CSP-60) not detected in actively growing endothelial cells or in cell types growing in multiple layers

(fibronectin/lactoperoxidase/fibroblast growth factor/cell–cell interaction)

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ABSTRACT **The formation of a highly organized vascular and corneal endothelial cell monolayer is associated with the appearance of a 60,000-dalton cell surface protein (CSP-60) (30,000 daltons after reduction with dithiothreitol) which is not detectable in rapidly growing endothelial cells and in subconfluent cultures that do not yet exhibit the strict morphology of a confluent monolayer. It is also absent from vascular smooth muscle cells and from endothelial cultures that are maintained in the absence of fibroblast growth factor and grow on top of each other at confluence. After disorganization of cells in a confluent endothelial monolayer by urea, EDTA, or trypsin, CSP-60 is no longer exposed on the cell surface, but it reappears as soon as the cells readopt their characteristic two-dimensional configuration. This reorganization can be achieved in the presence of cycloheximide and despite removal of fibronectin by urea, EDTA, or trypsin. Maximal amounts of fibronectin and no CSP-60 are detected in subconfluent, but not yet organized, endothelial cultures or in endothelial cells that no longer form a monolayer of nonoverlapping cells at confluence. Likewise, cultures of vascular smooth muscle cells contain fibronectin but no CSP-60. These results suggest that CSP-60, rather than fibronectin, could be involved in the adoption of a monolayer configuration by confluent endothelial cells.**

Vascular endothelial cells can be passaged at low densities and maintained for over 200 generations in tissue culture, provided fibroblast growth factor (FGF) is included in the culture medium (1, 2). Upon reaching confluence, the cultures adopt morphological appearance and differentiated properties similar to those observed *in vivo* (1–3). They form a monolayer of highly flattened, closely apposed, and nondividing cells, which produce an extracellular matrix composed mostly of a fibronectin–collagen meshwork (2–6). This leads to a significant decrease in the lateral mobility of cell surface receptors (4) and to subsequent formation of an endothelial barrier against receptor-mediated uptake of ligands such as low-density lipoprotein (7, 8). Cultured endothelial cells could, therefore, serve as a model for studying the molecular basis of cell–cell interaction associated with adoption of a monolayer configuration.

Components of the cell surface are involved in cell–cell interactions, and changes in the cell membrane have been shown to be associated with alterations in growth control, morphology, and adhesive properties brought about by both malignant cell transformation and normal differentiation (9–12). Of the few surface components studied to date, fibronectin is widely thought to play a leading role in the regulation of cell morphology, adhesive properties, and contact inhibition of migration (13–15). We have now studied how the presence of fibronectin and other specific cell surface proteins correlates with changes in cell density and whether alterations in surface proteins are induced by disorganization and subsequent reorganization of a highly confluent endothelial cell monolayer. Other cell types used for comparison are corneal endothelial cells, which both *in vivo* and *in vitro* form at confluence a monolayer of highly flattened and hexagonal cells (2, 16), and cells that grow in multiple layers, such as vascular aortic smooth muscle cells and a strain of vascular endothelial cells that were no longer maintained with FGF (7).

MATERIALS AND METHODS

Cells. Bovine endothelial and smooth muscle cells were obtained from the fetal heart and aortic arches of adult animals (1, 2). Bovine corneal endothelial cells were obtained from steer eyes (16). Cells were cloned and cultured in Dulbecco's modified Eagle's medium as described (1) 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 a gelatin-Sepharose column as described (17). Endothelial cells were passaged weekly at a 1:64 split ratio, and FGF (100 ng/ml) was added every other day until the cells were nearly confluent. The presence of Factor VIII antigen and the adoption of a strict monolayer configuration at confluence have been constant features of all subcultures of vascular endothelial cells (1–3). Cultures were used when sparse, subconfluent, or 8 to 10 days after reaching confluence.

Iodination of Cell Surface Proteins. Lactoperoxidase–glucose oxidase catalyzed radioiodination of cell monolayers was carried out in phosphate-buffered saline in the presence of $Na^{131}I$ (Amersham), lactoperoxidase (Calbiochem), and glucose oxidase (Sigma) as described (18). Iodinated cells were washed five times with Ca^{2+}, Mg^{2+}-free phosphate-buffered saline and lysed in buffer containing 15% glycerol, 2% sodium dodecyl sulfate, 75 mM Tris·HCl (pH 6.8), 2 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, 1 mM *N*-ethylmaleimide, and 1 mM iodoacetic acid (19). To reduce disulfide bonds, dithiothreitol (DTT) was added at 0.1 M before the samples were boiled (19).

Polyacrylamide Gel Electrophoresis. Samples containing 50,000–100,000 protein-bound cpm were applied to exponential gradient (6–16%, unless stated otherwise) polyacrylamide slab gels with a 3% stacking gel (20). For analysis in two dimensions (21), 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% (wt/vol) agarose in electrophoresis buffer. For reduction prior to the second dimension, the agarose contained 5% 2-mercaptoethanol. After

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Abbreviations: Dal, dalton; CSP-60, 60,000-Dal cell surface protein; DTT, dithiothreitol; FGF, fibroblast growth factor.

electrophoresis, the slab gels were fixed in 7% (wt/vol) acetic acid, dried onto filter paper, and subjected to autoradiography on Kodak NS-2T x-ray film for 8–24 hr.

RESULTS

Lactoperoxidase iodination of sparse and confluent cultures of vascular aortic endothelial and smooth muscle cells

The cell surface protein iodination pattern of either confluent or sparse vascular endothelial cell cultures was analyzed by gel electrophoresis before or after reduction with DTT. Although a number of differences in labeling intensities of several surface proteins are noted (Fig. 1), two components [larger than 400,000 and 60,000 daltons (Dal) before reduction, 230,000 and 30,000 Dal after reduction with DTT] appear to be highly susceptible to iodination by lactoperoxidase in confluent as opposed to sparse actively growing endothelial cells. Both the large and small components were capable of forming disulfide-bonded complexes, because reduction with DTT was associated with their disappearance from their original unreduced position and an appearance of two smaller components (230,000 and 30,000 Dal). Most of the high molecular weight material, which under nonreduced conditions barely penetrated the running gel (Fig. 1 *left*, lane A), was identified as crosslinked fibronectin by indirect immunoprecipitation with antifibronectin antiserum. Reduction with DTT greatly decreased the amounts of these

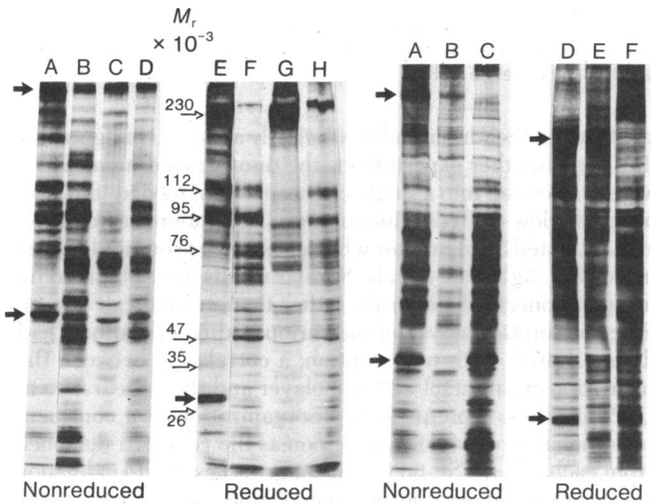

FIG. 1. (*Left*) Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of iodinated sparse and confluent cultures of bovine aortic endothelial and smooth muscle cells. Washed cells were radioiodinated, lysed, and analyzed by gradient (6.5%–16%) gel electrophoresis either before (lanes A–D) or after (lanes E–H) reduction with 0.1 M DTT. Endothelial cells: Confluent (lanes A and E) and sparse (lanes B and F). Bovine aortic smooth muscle cells: Confluent (lanes C and G) and sparse (lanes D and H). Gels were standardized with T4 phage [^{35}S]methionine-labeled proteins, and arrows mark the positions of fibronectin and CSP-60. (*Right*) Effect of urea and mild trypsinization on the appearance of CSP-60 and fibronectin in confluent monolayers of endothelial cells. Vascular endothelial monolayers, 3 weeks after reaching confluence, were treated with either urea (1 M in medium, 1 hr, 37°C) or trypsin (Worthington, 0.2 μg/ml, 1 hr, 37°C), washed, and iodinated. Samples were applied to a gradient (5–16%) polyacrylamide slab gel either before (lanes A–C) or after (lanes D–F) reduction with 0.1 M DTT. (Lanes A and D) Untreated, highly organized endothelial monolayer. Arrows mark the positions of fibronectin and CSP-60. (Lanes B and E) Confluent endothelial monolayers disrupted by urea into single round cells. Note the absence of CSP-60. The fibronectin of the extracellular matrix becomes exposed for iodination. (Lanes C and F) Confluent cell monolayer iodinated after mild trypsinization, which did not disorganize the monolayer configuration of the culture. Little or no fibronectin is left, but there is no effect on CSP-60.

large protein complexes and yielded a major band with an apparent molecular weight corresponding to that of fibronectin monomers (230,000) (lane E). Both the larger-than-400,000-Dal and 230,000-Dal components were highly sensitive to proteolysis and were removed from the upper cell surface by mild trypsinization (Fig. 1 *right*, lanes C and F) in agreement with reports (13–15) regarding the trypsin sensitivity of fibronectin. Samples derived from sparse endothelial cell cultures, as opposed to confluent cultures, contained almost none or much smaller amounts of fibronectin (*left*, lanes B and F).

The other major component which seems to be characteristic of confluent endothelial cells had an apparent M_r, before and after reduction with DTT, of about 60,000 and 30,000, respectively (*left*, lanes A and E). This component could not be identified with protein stains and was not affected by mild trypsinization, which removed the fibronectin but did not disrupt the cell monolayer (*right*, lanes C and F).

To determine whether the presence of both high and low molecular weight components (230,000 and 30,000, under reduced conditions) is a characteristic property of cells that form a highly organized cell monolayer, we also examined the surface iodination pattern of vascular smooth cells, which grow in multiple layers. As shown in Fig. 1 *left* (lanes C, D and G, H), samples derived from both confluent and sparse cultures of smooth muscle cells contained large amounts of disulfide crosslinked fibronectin but none of the 60,000-Dal (lanes C and D) or 30,000-Dal (lanes G and H) components. These results therefore suggest that the 60,000-Dal component, which we have named CSP-60 rather than fibronectin, might be a component specific to the adoption of the flattened and closely apposed morphology characteristic of quiescent endothelial cells.

Two-dimensional electrophoresis of lactoperoxidase iodinated cells

To determine whether the 30,000 M_r component is present in its unreduced form as a dimer or is disulfide bonded to a higher molecular weight surface component such as fibronectin, we analyzed iodinated surface proteins on two-dimensional sodium dodecyl sulfate gels. These gels were run unreduced in the first dimension and reduced in the second (21). Fig. 2*A* demonstrates that samples derived from a confluent vascular endothelial monolayer contained a major off-diagonal spot with an apparent M_r of about 30,000, which migrated as a 60,000-Dal component on the unreduced dimension. A lactoperoxidase iodinable component that has similar electrophoretic properties also appeared in samples derived from confluent corneal endothelial cultures that show a closely apposed, strictly hexagonal organization of cells (2, 16) (Fig. 2*B*). In contrast, there was no such off-diagonal 30,000 M_r component in samples derived from sparse, actively growing endothelial cell cultures (Fig. 2*C*) or from either confluent (Fig. 2*D*) or sparse cultures of smooth muscle cells. In most cases, two minor off-diagonal spots were detected on either side of the major CSP-60 spot, and these also appeared in samples derived from cultures that lacked CSP-60. Control gels that were run either unreduced or reduced in both directions showed no such off-diagonal spots. These results again demonstrate that the 60,000-Dal protein (CSP-60) is unique to confluent endothelial monolayers and is present on the cell surface as a disulfide-bonded complex, possibly a dimer composed of 30,000-Dal monomers. In addition, a higher proportion of other intermolecular disulfide-bonded complexes, in particular of crosslinked fibronectin, was found in samples derived from confluent than from sparse cultures of vascular endothelial cells (Fig. 2*A* and *C*). This might contribute to the restriction of surface receptor lateral mobility observed in confluent endothelial cell monolayers (4). No 60,000-Dal component (which

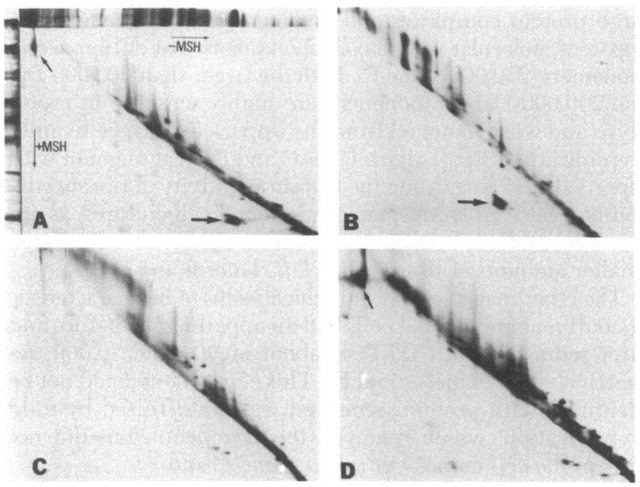

FIG. 2. Two-dimensional gel analysis of lactoperoxidase iodinated cells. Cultures were iodinated and the cells were lysed and harvested under nonreducing conditions. Aliquots were run on a gradient (6.5–16%) polyacrylamide slab gel, and the gel was cut into appropriate narrow strips that were then equilibrated with 5% 2-mercaptoethanol (MSH) and each placed at the top of another 6.5–16% slab gel. (*A*) Confluent monolayer of vascular endothelial cells. Arrows mark the position of fibronectin (upper left) and CSP-60 (lower right). (*B*) Confluent corneal endothelial cell monolayer. CSP-60 appears as a major off-diagonal spot. Fibronectin is hardly exposed for iodination. (*C*) Sparse, actively growing vascular endothelial cells. Note the absence of fibronectin and CSP-60. (*D*) Confluent vascular smooth muscle cells. Note the absence of CSP-60 and presence of fibronectin.

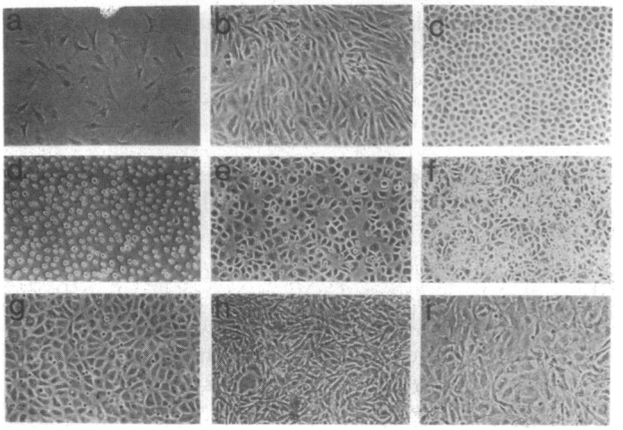

FIG. 3. Phase contrast micrographs of disorganized and reorganized monolayers of vascular endothelial cells. (*a*) Sparse, actively growing culture 2 days after seeding. (*b*) Subconfluent culture 5 days after seeding. (*c*) A confluent cell monolayer 7 days after reaching confluence. (*d*) A confluent cell monolayer treated with urea (1 M in medium, 1 hr, 37°C). (*e*) Urea-treated cells at an intermediate stage of reorganization, 1 hr after washing the urea out and incubating the culture under growth conditions. (*f*) Urea-treated cells seeded at high density and observed after 5 hr. 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 hr later after washing out the excess cells. (*h*) Cells from a sparse culture that were pooled together, reseeded at a high density, and observed 48 hr 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.

after reduction yielded a 30,000-Dal component) was detected either in the serum proteins, which adsorbed to the tissue culture plastic, or in the lactoperoxidase reaction mixture in the absence of cells. There was also no change in CSP-60 in the gel pattern of confluent endothelial cells maintained with 10% serum for the period of active growth and then in a medium containing 0.1% serum for 48 hr prior to labeling. Lactoperoxidase iodination of medium containing 10% calf serum did not reveal the existence of a 60,000-Dal component that yielded a 30,000-Dal component after reduction. These results demonstrate that CSP-60 is of cellular origin rather than a protein that might be specifically absorbed to confluent endothelial cell monolayers. FGF did not induce the appearance of CSP-60 in sparse or confluent cultures of smooth muscle cells, but its addition to growing endothelial cells was required for both the appearance of CSP-60 and the adoption of a monolayer configuration at confluency.

Disorganization and reorganization of a highly confluent endothelial cell monolayer and its effect on the appearance of fibronectin and CSP-60

To further study the respective roles of fibronectin and CSP-60 in controlling the configuration that endothelial cells adopt in culture, we have looked for changes in the lactoperoxidase iodination of these proteins associated with the disorganization and subsequent reorganization of a confluent vascular endothelial cell monolayer. Disorganization of the cell monolayer was brought about by exposing confluent and highly organized endothelial cultures to urea (Fig. 3), trypsin, or EDTA. In all cases, soon after the addition of the disruptive agent cells started to detach from each other and appeared as single spheres that were still attached to the substratum (Fig. 3*d*). Cultures were then washed free of the disruptive agent and iodinated. Alternatively, disorganized cultures were incubated in medium supplemented with a fibronectin-depleted serum for 2–5 hr prior to labeling. This procedure allowed the cells to reorganize and to adopt their initial cuboidal, closely apposed configuration (Fig. 3*e*). In other experiments, the disorganized monolayers were dissociated into a single cell suspension, seeded into new plates at a low (1:10 dilution) or high (no dilution) cell density, and iodinated 5–24 hr later when either sparse or highly organized (Fig. 3*g*), respectively. Similar results were obtained by using fibronectin-free serum rather than normal calf serum. Electrophoretic analysis of the various iodinated cultures had demonstrated, with no exception, a correlation between the formation of an intact cell monolayer and the appearance of CSP-60. Thus, subsequent to disorganization of a confluent endothelial monolayer and as soon as the cells had detached from each other, CSP-60 was no longer accessible to iodination by lactoperoxidase (Fig. 4, lanes E and J). In contrast, the adoption of a highly organized configuration 2–5 hr after washing the disruptive agents (lanes B, F, and K) or 5–12 hr after reseeding the cells at their original high density (lanes C, G, and L) was, regardless of the disruptive agent used, always associated with the reappearance of CSP-60 as a major cell surface protein accessible to iodination. CSP-60 was, however, largely or almost entirely missing from the surface of cells that were taken from a disorganized monolayer, reseeded at a low density, and iodinated prior to the formation of a confluent cell monolayer (Fig. 4, lanes D, H, and M). These cells adhere and spread perfectly well on the tissue culture dish and adopt, even at a low density, a flattened morphology. Thus, the appearance of CSP-60 was not associated with substrate adhesion and flattening of cells, but it did correlate with the formation of an organized monolayer composed of nonoverlapping cells. The reappearance of CSP-60 in reconstituted monolayers that were first disorganized by trypsin, EDTA, or urea was not affected by exposing the endothelial cell monolayer to 0.1 mM cycloheximide 1 hr prior to its disruption and during the 2- to 5-hr time period that it took to get a reorganized monolayer. Because this concentration of cycloheximide inhibited protein synthesis

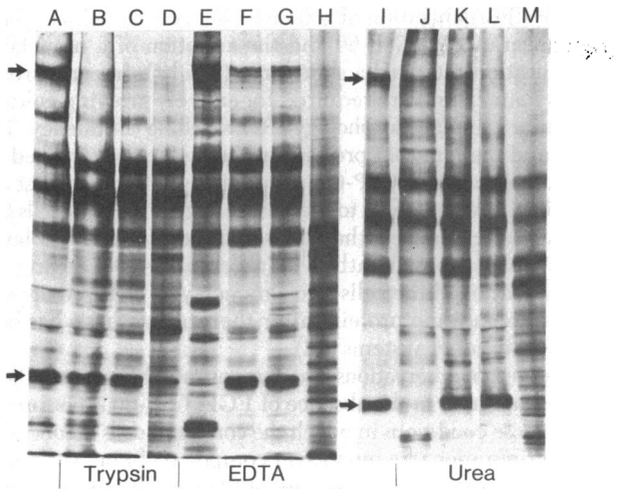

FIG. 4. Appearance of CSP-60 and fibronectin after disorganization and a subsequent reorganization of a confluent cell monolayer. Confluent endothelial monolayers were treated with either trypsin [0.05% (GIBCO), 3 min, 37°C], EDTA (0.03% in Ca^{2+}, Mg^{2+}-free phosphate-buffered saline, 30 min, 37°C), or urea (1 M in medium, 1 hr, 37°C) to dissociate cell-to-cell contacts. The disruptive agent was then washed out and the cells were allowed to reorganize in medium containing a fibronectin-depleted serum on the same plate or were reseeded at a high or low density. Disorganized and reorganized cultures were iodinated and analyzed by gel electrophoresis after reduction of the samples with DTT. Lanes: (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 5 hr under growth conditions to readopt its original monolayer configuration. (C) Cells 12 hr after trypsinization and seeding at a high density. The cells fully adopted a monolayer organization and show the presence of CSP-60, but little or no fibronectin. (D) Cells 12 hr 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 hr under growth conditions to readopt its original morphology. (G) Cells 12 hr after EDTA-dissociation and seeding at a high density. (H) Cells 12 hr 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-hr incubation under growth conditions. (L) Cells 24 hr after exposure to urea and reseeding at a high density. (M) Cells 24 hr after treatment with urea and reseeding at a low density.

by more than 90%, it is demonstrated that the reappearance/exposure of CSP-60 in cells that reorganize into a monolayer does not require protein synthesis. ^{131}I-Labeled CSP-60 was present in a confluent cell monolayer that was first iodinated and then treated with urea, suggesting that CSP-60 was not released but rather became inaccessible for iodination in a disorganized cell monolayer.

After removal of the disorganizing agents by either washing (Fig. 4, lanes B, F, and K) or replating the cells at high density in the presence of fresh medium containing fibronectin-depleted serum (lanes C, G, and L), the cells reorganized into a monolayer. As demonstrated in Fig. 4, this can be accomplished without the reappearance of fibronectin, which was largely removed from the cell surface by trypsin, EDTA, or urea, thereby indicating that fibronectin does not play a crucial role in this reorganization. There was also no detectable deposition of fibronectin on the tissue culture plastic during the 5- to 12-hr period required for the reconstitution of an endothelial cell monolayer after disorganization and reseeding the cells at high density. This further suggests that fibronectin is not essential for the formation of a tightly packed endothelial cell monolayer.

Appearance of fibronectin and CSP-60 in confluent endothelial cultures that are not organized into a monolayer of closely apposed cells

Sparse Cells, Pooled and Reseeded at a High Density. In order that we might further study the correlation between the appearance of CSP-60 and the formation of a confluent cell monolayer, cells from sparse, actively growing cultures that lack CSP-60 were dissociated with either trypsin or EDTA, pooled, and reseeded at a density (900 cells per mm^2) characteristic of a highly organized cell monolayer. These cells adhered and spread on top of each other and formed multilayers (Fig. 3*h*). Even after 72 hr in a medium containing either normal or fibronectin-free calf serum, the cultures still failed to adopt the closely apposed morphology of a confluent cell monolayer. This is in contrast to the behavior of cells that were taken from a dissociated cell monolayer and reseeded at a similarly high density. These cells not only showed a rapid (5–12 hr) rate of reorganization into a monolayer, but the excess cells remained round and did not adhere or spread on top of cells that were already attached to the tissue culture dish (Fig. 3*f*). Electrophoretic analysis of iodinated cell surface proteins from the resulting unorganized or reorganized confluent cultures revealed the presence of CSP-60 in cells initially taken from confluent (Fig. 5, lane E), but not from sparse (lane D), cultures. In this respect, sparse cells that were seeded at a high density behave as do subconfluent endothelial cultures, both showing an unorganized morphology (Fig. 3 *b* and *h*), little or no CSP-60, and large amounts of fibronectin (Fig. 5, lanes B and D).

Confluent Vascular Endothelial Cells Growing on Top of Each Other. Vascular endothelial cells no longer maintained with FGF lose within 2 or 3 passages their unique morphological organization at confluence, become considerably larger, and grow on top of each other (Fig. 3*i*) (7). Lactoperoxidase iodination of these cultures revealed either no CSP-60 or only small amounts of it in comparison with a confluent, normal endothelial cell monolayer (Fig. 5, lane F). The residual amounts of CSP-60 could have originated from areas in the culture still exhibiting a monolayer-like morphology (Fig. 3*i*). By using either antifibronectin and immunofluorescence or the lactoperoxidase iodination technique (Fig. 5, lane F), we saw that the overlapping endothelial cells contained, even late at confluency, large amounts of fibronectin distributed in thin

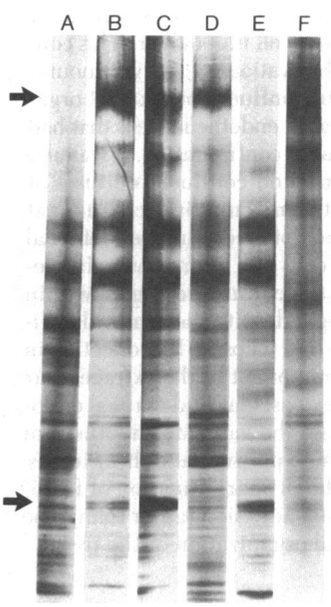

FIG. 5. Appearance of fibronectin and CSP-60 in organized and nonorganized cultures of endothelial cells. Cultures were radioiodinated and samples were analyzed by gel electrophoresis after reduction with DTT. Lanes: A, sparse, actively growing cells; B, subconfluent culture; C, confluent cell monolayer; D, cells derived from sparse cultures reseeded at a high density and iodinated 48 hr later; E, cells 12 hr after trypsinization of a confluent monolayer and reseeding at a high density; F, confluent but unorganized endothelial culture maintained for three passages in the absence of FGF.

fibers over the entire apical cell surface area. Endothelial cells that have lost their ability to adopt a monolayer configuration resembled either subconfluent cultures or sparse endothelial cells reseeded at a high density, in that large amounts of fibronectin but little or no CSP-60 were detected.

DISCUSSION

Confluent cultures of vascular endothelial cells mimic their *in vivo* counterparts in their two-dimensional organization, polarity, and barrier function (1–8). The vascular endothelium, therefore, provides a system with which to study whether changes in the cell surface correlate with the ability of the endothelial cells to adopt a monolayer configuration. 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 cell density, dissociation, and subsequent formation of cell-to-cell contacts and by a change in growth properties that allows the endothelial cells to grow on top of each other. Our results indicate a correlation between the formation of a highly organized endothelial cell monolayer and the appearance of a 60,000-Dal component, CSP-60. This correlation is based on the following findings. (*i*) Under various experimental conditions, the adoption of a closely apposed and highly organized morphology was associated with the appearance of CSP-60 as a major cell surface component susceptible to iodination by lactoperoxidase in both vascular and corneal endothelial cells. This occurred under normal conditions—i.e., as soon as the culture adopted the configuration of a confluent cell monolayer or in reconstituted monolayers derived from disorganized cultures. (*ii*) In the various cultures studied so far, CSP-60 was not detected under sparse conditions, when no contact between the cells existed, or at a subconfluent density, when cells contacted each other but were still elongated, overlapped each other, and were not yet organized in a tightly packed cuboidal manner. (*iii*) Cells that grow in multiple layers at confluence (i.e., smooth muscle cells and a vascular endothelial cell strain obtained by culturing the cells in the absence of FGF) contained no CSP-60 even after being maintained at confluence for an extended period of time. (*iv*) CSP-60 was not detectable in a disorganized cell monolayer or prior to the formation of an intact cell monolayer after reseeding the confluent cells at a low density, nor could it be found in sparse cells that were pooled together and reseeded at a high density to yield a confluent but unorganized endothelial culture. The present results also demonstrate that fibronectin, unlike CSP-60, is not essential for the formation of a highly organized cell monolayer. This conclusion is based on the following observations: (*i*) Large amounts of fibronectin were detected in subconfluent but not yet organized endothelial cultures as well as in endothelial cells that had lost their ability to adopt a monolayer configuration. It was also present in large amounts of sparse and confluent cultures of vascular smooth muscle cells that form a multiple cell layer at confluence. (*ii*) After trypsinization of a confluent endothelial monolayer and reseeding of the cells at a high density in medium containing no fibronectin, the cultures resumed, within 5–12 hr, their original morphology, despite the removal of fibronectin from the cell surface by trypsin. Fibronectin, in contrast to CSP-60, is a major component of the extracellular matrix produced at confluence by vascular and corneal endothelial cell cultures (2–6). Because the vascular endothelium of the big arteries is subjected to high pressure and sheer forces, it is 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.

After disorganization of a preexisting cell monolayer, both the appearance of CSP-60 and the adoption of a monolayer configuration required a short time (2–5 hr). In comparison, no less than 72 hr were required for actively growing cultures to acquire a similar morphology after reaching confluency. This result and the fact that protein synthesis was not required for the reappearance of CSP-60 in a monolayer that was first disrupted and then allowed to reorganize suggest that, in cells that 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. Delayed appearance of membrane proteins after their biosynthesis has been observed in other systems (22).

The present observations also demonstrate that, in endothelial cells maintained in the absence of FGF, CSP-60 is not present under those conditions in which the confluent cells do not adopt the configuration of a monolayer composed of nonoverlapping cells. Because various properties of the vascular endothelium, *in vivo* as well as *in vitro*, are dependent on the adoption of a monolayer configuration, CSP-60, which is only present in cells that exhibit this morphology, could be considered to be one of the determinants in the expression of the various differentiated properties that are revealed by confluent cultures of vascular or corneal endothelial cells (1–8).

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