Proc. Natl. Acad. Sci. USA
Vol 75, No. 7, pp. 3273–3277, July 1978
Cell Biology

Identification, localization, and role of fibronectin in cultured bovine endothelial cells

(vascular endothelium/extracellular matrix/cell adhesion/thrombosis/cold-insoluble globulin)

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Communicated by Donald A. Glaser, March 10, 1978

ABSTRACT **We have examined bovine aortic endothelial cell cultures for the presence of fibronectin, a high molecular weight cell-surface glycoprotein. Sparse cultures contain fibronectin only on dorsal cell surfaces at regions of cell–cell contact, as detected by immunofluorescence. In contrast, when the endothelial cells reached confluence as a highly contact-inhibited monolayer, fibronectin was detected in an extracellular matrix underneath the cell monolayer but not on top of the monolayer. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of isolated extracellular matrix revealed that a predominant component of the matrix is a protein of approximately 2.3×10^5 molecular weight, which has been identified as fibronectin.**

The vascular endothelium exists as a monolayer of highly flattened and contact-inhibited cells; because of their location at the interface between blood and tissue, endothelial cells are the chief elements involved in the permeability of blood vessels (1, 2). Although the side of the vascular endothelium exposed to the bloodstream is nonthrombogenic, the subendothelial matrix on which the cells rest is very thrombogenic (3, 4). Thus, a disruption of the vascular endothelium can expose the underlying basement membrane, resulting in the aggregation of platelets and thrombus formation. Therefore, factors that are involved in the attachment of endothelial cells to the basement membrane are very important for the proper functioning of the vascular endothelium.

Biochemical analyses have established that basement membranes possess a highly cross-linked form of collagen (5), rendering basement membranes completely insoluble under physiological conditions. Although most biochemical studies of basement membranes have dealt with the structure and synthesis of collagen, noncollagenous matrix glycoproteins have also been found in some basement membranes (6). Recent studies indicate that one of these glycoproteins may be identical to fibronectin (7), a major cell-surface glycoprotein that is immunologically identical to cold-insoluble globulin (8), a plasma protein which is presumably the plasma form of fibronectin that is shed from cells into the blood. Fibronectin appears to be similar, if not identical, to the large external transformation-sensitive (LETS) protein, which exists on the surface of certain untransformed cells, but not on the surface of transformed cells (9–11). Fibronectin is composed of two subunits of molecular weight $2.15–2.30 \times 10^5$ linked by disulfide bonds (12, 13). Linder *et al.* (14) recently showed by immunofluorescence studies that fibronectin is found in the connective tissue and basement membranes of several organs in the developing chick embryo. Because of recent evidence that fibronectin is involved in cell–cell and cell–substrate adhesion (15–18), possibly by interacting with collagen (19, 20), we have been studying the association of fibronectin with the extracellular matrix of vascular endothelial cells. In this paper we report on the formation of basement membrane-like material (extracellular matrix) in a cloned line of adult bovine aortic endothelial (ABAE) cells that we have recently characterized (21), and we also show that fibronectin is a major component of the extracellular matrix produced by ABAE cells.

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METHODS

Cell Culture. The cloning, culturing, and characterization of ABAE cells have been described (21). ABAE cells are identified as endothelial cells on the basis of their morphology and ultrastructure and on their ability to synthesize Factor VIII (antihemophilic factor antigen), a marker for endothelial cells (22). These cells are routinely maintained in fibroblast growth factor (23).

Electron Microscopy. Cells were prepared for electron microscopy by described methods (24), and then examined in a Hitachi S-500 scanning electron microscope or a Hitachi 12-A transmission electron microscope.

Indirect Immunofluorescence Microscopy. Cells were grown on 2.5-cm round glass coverslips for immunofluorescence studies. Some cultures were extracted with 0.5% Triton X-100 in phosphate-buffered saline (10 min at room temperature with gentle shaking) before labeling to remove the cell monolayer (Fig. 1). At various times in culture the coverslips were washed four times with 4-(2-hydroxyethyl)-1-piperazineethanesulfonate (Hepes)-buffered Dulbecco's modified Eagle's medium containing 0.5% bovine serum albumin. They were then treated for 20 min at room temperature with a $1/40$ dilution of rabbit antiserum against human plasma fibronectin (a gift from Eva Engvall, City of Hope, Duarte, CA) in the same medium. This antiserum gives one precipitation band against whole human serum by immunoelectrophoresis and crossreacts with fibronectin from several species (25). After four more washes in medium the coverslips were treated for 20 min at room temperature with a $1/100$ dilution of rhodamine-labeled goat anti-rabbit IgG (Cappel) in the same medium. In some experiments cells were fixed with 2% paraformaldehyde/0.1 M cacodylate (pH 7.2) for 20 min at room temperature before they were labeled with antiserum. Prefixing the cells did not change the experimental results, so most labeling experiments were performed with live cells. Purified human plasma fibronectin was also a gift from Eva Engvall. Fluorescence was visualized on

Abbreviations: ABAE cells, adult bovine aortic endothelial cells; Na-$DodSO_4$, sodium dodecyl sulfate.

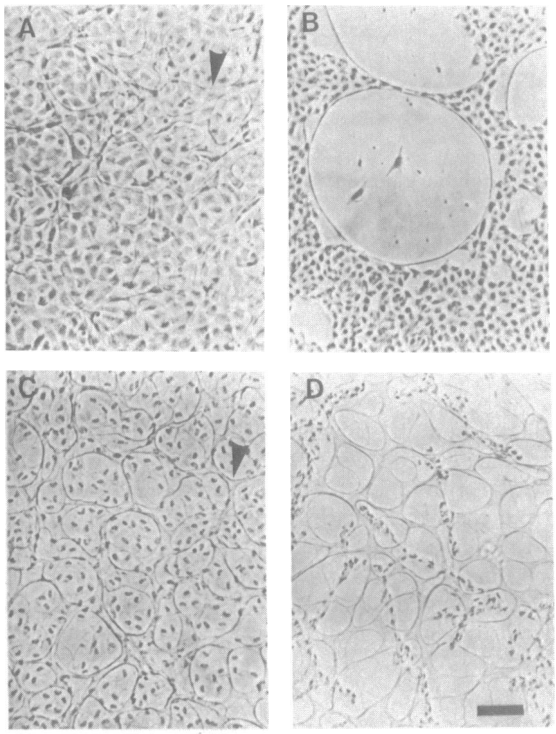

FIG. 1. Configuration of a monolayer of ABAE cells after long-term culture. ABAE cell cultures were seeded and maintained as described in the text. One week after reaching confluence, an extracellular matrix (arrow) was visible under the monolayer (*A*); by 2 weeks certain regions of the monolayer retracted, forming circles that were limited internally by a membrane (*B*). When 2-week-old monolayers were treated with 0.5% Triton X-100 (*C* and *D*), the monolayer instantly "dissolved" (*C*), leaving behind nuclei and the extracellular matrix (arrow). After a 5-min exposure to 0.5% Triton X-100, most of the nuclei had floated away from the extracellular matrix (*D*), which was two or three layers deep in some areas. Bar equals 50 μm.

a Zeiss fluorescent microscope equipped with epi-illumination, and photographs were taken on Kodak Tri-X film.

Radioactive Labeling of Cells and Extracellular Matrix. For metabolic labeling of cell cultures, ABAE cells were grown to confluence in 3.5-cm tissue culture dishes (Falcon). After 12–14 days in culture (approximately 1 week after reaching confluence), 75 μCi of [^{35}S]methionine (New England Nuclear; 250 Ci/μmol) was added to the culture for 2 days, and the cell monolayers were then washed three times with phosphate-buffered saline before further processing. For $Na^{125}I$–lactoperoxidase-catalyzed iodination of extracellular matrix, ABAE cells were grown to confluence in 10-cm tissue culture dishes. After 2 weeks in culture the cell monolayers were washed three times in phosphate-buffered saline and then treated with 0.5% Triton X-100 in phosphate-buffered saline for 10 min at room temperature with gentle shaking. This treatment removes the cell monolayer from the dish with little remaining cell debris, but the extracellular material is left on the dish. The detergent-treated dishes were then washed three times with phosphate-buffered saline, and the extracellular material remaining on the dish was labeled with 0.5 mCi of $Na^{125}I$ (New England Nuclear; 14 mCi/μg) in the presence of 100 μg of lactoperoxidase (Sigma) and 0.002% H_2O_2, as previously described (26).

Sodium Dodecyl Sulfate ($NaDodSO_4$)/Polyacrylamide Gel Electrophoresis. Gel electrophoresis was performed by using a discontinuous buffer system (27) with a 4% acrylamide stacking gel and a 7.5% acrylamide running gel. Samples were dissolved in solubilizing buffer (2% $NaDodSO_4$/2% 2-mercaptoethanol/10% glycerol/50 mM Tris·HCl at pH 7) and boiled for 3 min; extracellular matrix remaining on the dish was scraped into the solubilizing buffer. After electrophoresis the gels were stained for proteins with Coomassie brilliant blue, dried, and subjected to autoradiography. The following proteins were used for molecular weight standards: rat myofibril actin (4.3×10^4); rabbit IgG heavy chain (5.5×10^4); bovine serum albumin (6.8×10^4); β-galactosidase (1.35×10^5); and rat myofibril myosin (2.0×10^5).

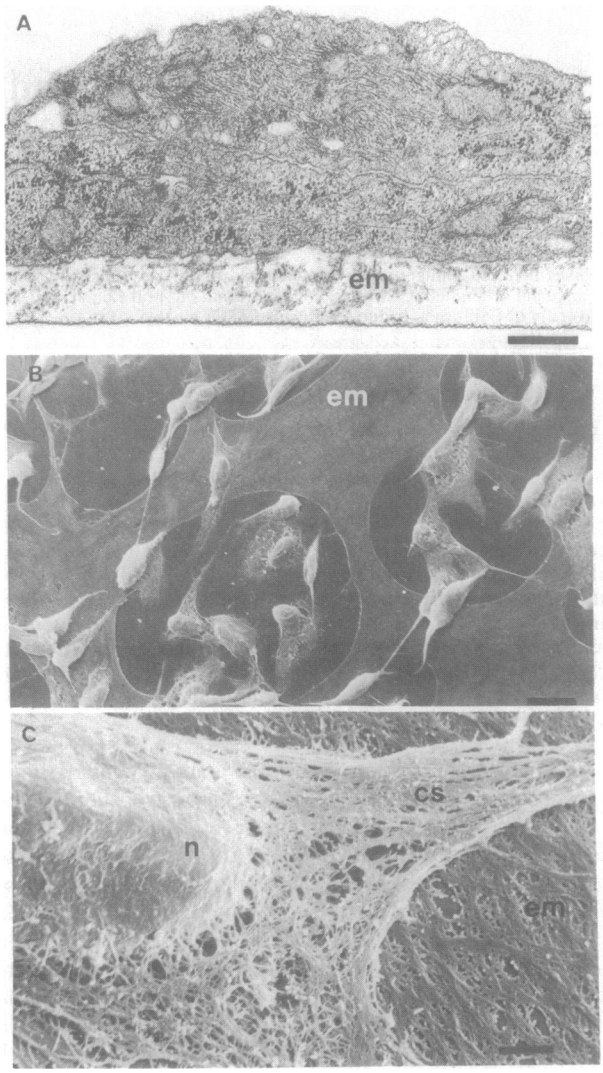

FIG. 2. Electron micrographs of 2-week-old ABAE cultures. (*A*) Transmission electron micrograph of a thin section of an ABAE monolayer cut perpendicular to the monolayer showing the extracellular matrix (em) underneath the cells. The dark line along the bottom of the micrographs is the plastic substrate of the tissue culture dish. Bar equals 0.5 μm. (*B*) Scanning electron micrograph of an ABAE monolayer treated very briefly with 0.5% Triton X-100. The extracellular matrix (em) is quite apparent and many ABAE cytoskeletons are still attached to the substrate. Bar equals 25 μm. (*C*) Same as *B*, but showing a single ABAE cytoskeleton (cs) and attached nucleus (n) stretched over the extracellular matrix (em). Bar equals 2 μm.

RESULTS

When confluent monolayers of ABAE cells (2 weeks in culture) are examined by transmission electron microscopy, an extracellular matrix typical of a basement membrane *in vivo* is seen underneath the cell monolayer (Fig. 2*A*). This appears to be

similar to the production of an extracellular matrix observed in cultures of endothelial cells from human umbilical cord vein (28). We found that a better way to observe the extracellular matrix is to first remove the cell monolayer with 0.5% Triton X-100, a nonionic detergent that essentially dissolves cell membranes and releases most of the cytoplasmic components (Figs. 1 and 2 *B* and *C*). In certain cell types treated with Triton X-100 a cellular cytoskeleton is left undissolved by the detergent (29). Scanning electron micrographs of confluent monolayers of ABAE cells (2 weeks in culture) treated briefly with 0.5% Triton X-100 (1 min at room temperature) are shown in Fig. 2 *B* and *C*. Fig. 2*B* shows that the extracellular matrix appears to be produced as large sheets which adhere to the substrate. At this time in culture (2 weeks) parts of the substrate are devoid of the extracellular matrix; many ABAE cytoskeletons and intact nuclei remain attached to either the extracellular matrix or the substrate after brief treatment with Triton X-100. By 4 weeks most of the substrate is covered with an extracellular matrix. The matrix, viewed at higher magnification, is seen to consist of a very complex organization of extensively overlapping extracellular fibers, quite distinct from the organization of the ABAE cytoskeleton (Fig. 2*C*).

Since it has been shown that fibronectin is found in basement membranes from various organs of the chick embryo (14), it was of interest to see if fibronectin is a component of the ABAE extracellular matrix. Fig. 3 shows the results of indirect immunofluorescence studies on ABAE cultures by using a monospecific rabbit antiserum against human plasma fibronectin. When confluent monolayers (1–4 weeks in culture) of ABAE cells were examined by indirect immunofluorescence for fibronectin, fluorescence was localized to discrete areas (Fig. 3*A*) that corresponded to spaces between adjacent cells (Fig. 3*B*). Careful focusing revealed that this fluorescence results from fibronectin associated with regions of the extracellular matrix exposed in the intercellular spaces. Labeling live cells or cells prefixed with 2% paraformaldehyde did not change the results of these experiments. Fibronectin was not found associated with the cell surface exposed to the medium at any time after the cells became confluent (1–4 weeks in culture). In contrast, fibronectin was found on top of the cells at regions of cell–cell contact in sparse cultures.

The association of fibronectin with ABAE extracellular matrix was very apparent when confluent monolayers of ABAE cells were treated with 0.5% Triton X-100 for 10 min with shaking (to remove all the cells) before labeling for immunofluorescence (Fig. 3*C–F*). Since fibronectin is resistant to treatment with low concentrations of nonionic detergents (30), it probably remains firmly attached to the extracellular matrix during this treatment. Fig. 3*C* shows labeling of extracellular matrix in a 1-week culture, whereas Fig. 3*E* shows labeling in a 4-week culture. In both cases, extensive fibrillar labeling patterns are observed and the distribution of the label corresponds to the organization of the matrix seen in phase-contrast micrographs of the same fields (Fig. 3 *D* and *F*).

The fibrillar network of extracellular matrix containing fibronectin was very sensitive to trypsin. Treatment of this matrix with 0.2 μg of trypsin per ml for 10 min at 37° resulted in the loss of fluorescence for fibronectin. The fluorescence observed was specific, since fluorescence was not observed when normal rabbit serum was substituted for rabbit antifibronectin antiserum (Fig. 3*G*) or when purified human plasma fibronectin was incubated with the anti-fibronectin antiserum before labeling (Fig. 3*H*). It is unlikely that the fibronectin-specific fluorescence was due to calf serum fibronectin present in the culture medium which may have nonspecifically adhered to the substratum, since several lines of evidence indicate that fibronectin is of cellular origin (8, 18, 31). To conclusively establish the cellular origin of fibronectin used in these cultures, we removed fibronectin from calf serum by passing the serum through a gelatin-Sepharose affinity column, which quantitatively removes fibronectin from serum (32). The loss of serum fibronectin was established by immunoelectrophoresis of gelatin-treated calf serum against rabbit anti-human plasma fibronectin antiserum (which normally produces one precipitation band when run against untreated serum). When ABAE cells were grown in fibronectin-depleted serum and examined by immunofluorescence for fibronectin, the pattern of fluorescence was unchanged.

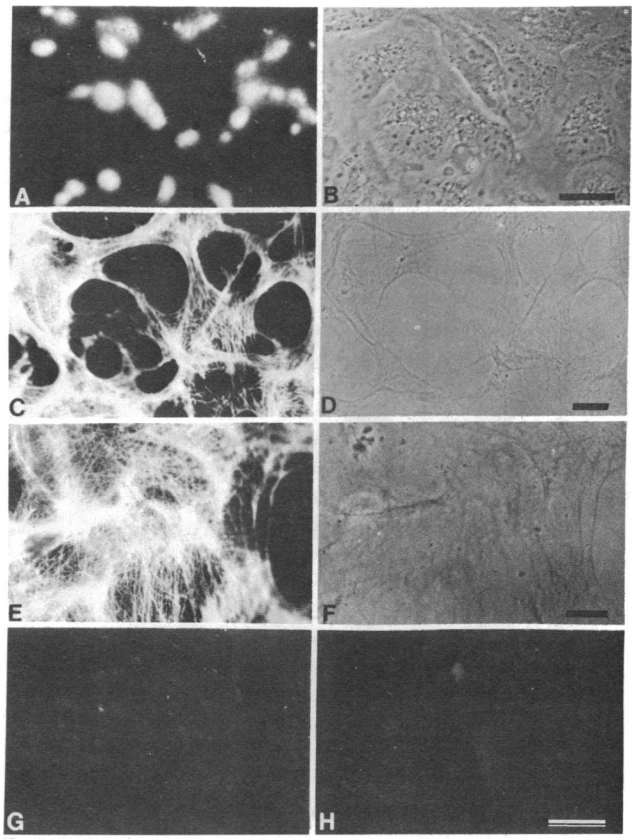

FIG. 3. Indirect immunofluorescence localization of fibronectin in ABAE cultures. (*A*) Staining of a 2-week-old culture without Triton X-100 treatment shows discrete areas of fluorescence, which correspond to intercellular spaces. (*B*) Phase-contrast micrograph of the same field in *A*; bar equals 20 μm. (*C*) Staining of 1-week-old culture after Triton X-100 treatment shows intense labeling of extracellular matrix. (*D*) Phase-contrast micrograph of the same field in *C*; bar equals 20 μm. (*E*) Same as *C*, except a 4-week-old culture was used. (*F*) Phase-contrast micrograph of the same field in *E*; bar equals 10 μm. (*G*) Control showing no fluorescence with normal rabbit serum on ABAE culture after Triton X-100 treatment. (*H*) Control showing inhibition of fluorescence with purified plasma fibronectin on ABAE culture after Triton X-100 treatment; bar equals 20 μm (same for *G*).

The protein components of the ABAE extracellular matrix were examined by solubilizing the components in $NaDodSO_4$ and then subjecting them to $NaDodSO_4$/polyacrylamide gel electrophoresis. When extracellular matrix prepared by 0.5% Triton X-100 treatment of cell monolayers was labeled by $Na^{125}I$–lactoperoxidase-catalyzed iodination and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis, a band that migrated in the position of fibronectin was heavily labeled (Fig. 4, slot A). Many other bands were also labeled, some of which

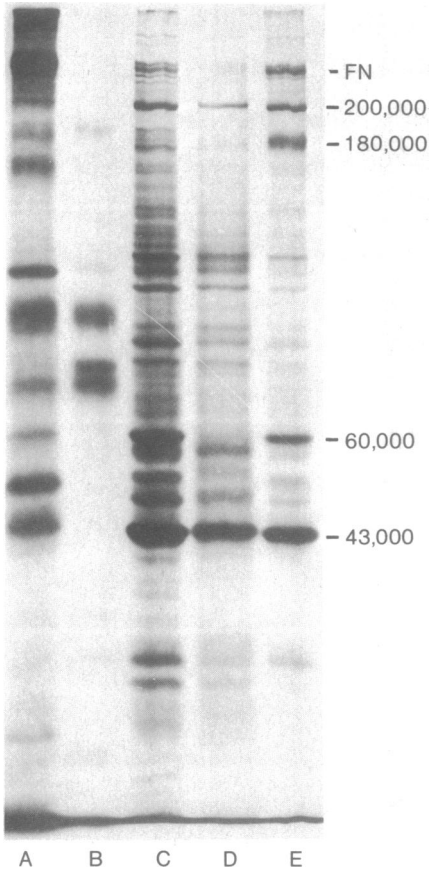

FIG. 4. Autoradiogram of $NaDodSO_4$/polyacrylamide gel of ABAE cultures. (Slot A) $Na^{125}I$–lactoperoxidase labeling of the extracellular matrix after a 2-week-old ABAE culture was treated with Triton X-100. (Slot B) A control plate to identify possible serum proteins attached to the extracellular matrix; tissue culture dishes without cells were incubated with medium containing 10% calf serum for 2 weeks, treated with Triton X-100, and labeled with $Na^{125}I$, as in A. (Slots C–E) Results of labeling ABAE cultures with [^{35}S]methionine followed by treatment with Triton X-100. (Slot C) Total cell protein control; (slot D) material removed by 0.5% Triton X-100; (slot E) extracellular matrix remaining on the dish after Triton X-100 treatment. Fibronectin (FN, 2.3×10^5 molecular weight), a protein of 1.8×10^5 molecular weight, and a protein of 6×10^4 molecular weight were preferentially found in the extracellular matrix, whereas the proteins of 2×10^5 and 4.3×10^4 molecular weight were found in both the detergent extract and the extracellular matrix.

are probably serum proteins (compare with 10% calf serum control, Fig. 4, slot B). The extracellular matrix was also examined on $NaDodSO_4$/polyacrylamide gels after metabolic labeling of ABAE monolayers with [^{35}S]methionine (Fig. 4, slots C–E) to eliminate contaminating serum proteins. Under the conditions used for metabolic labeling, approximately 15% of the total incorporated radioactivity was found in the extracellular matrix and there were five major labeled proteins in the extracellular matrix after extraction with 0.5% Triton X-100 (Fig. 4, slot E): a protein of 2.3×10^5 molecular weight that comigrates with fibronectin; a protein of 2.0×10^5 molecular weight that comigrates with rat myofibril myosin; a protein of 4.3×10^4 molecular weight that comigrates with rat myofibril actin; and proteins of 1.8×10^5 and 6.0×10^4 molecular weight. Many minor components were also evident in the autoradiograms (Fig. 4, slot E). The labeled protein profile of the ABAE extracellular matrix shown in Fig. 4, slot E, is probably oversimplified since this material probably contains crosslinked collagen (28), which would not be solubilized by the denaturing conditions used here. Also, the protein profile of the ABAE extracellular matrix may be contaminated with some intracellular cytoskeletal components, although the extracellular matrix preparations for these radiolabeling experiments were free of any ABAE cytoskeletons, as judged by scanning electron microscopy. However, it is clear that a major band that comigrates with fibronectin is a predominant component of the ABAE extracellular matrix remaining on the culture dish after treatment with Triton X-100 (Fig. 4, slot E), and this protein does not appear in the Triton X-100 extract of the ABAE monolayer (Fig. 4, slot D). Preliminary experiments comparing the tryptic peptide map of this band with that of purified fibronectin indicate that the protein of 2.3×10^5 molecular weight is indeed fibronectin.

DISCUSSION

We have shown that cloned ABAE cells produce large amounts of an extracellular matrix in tissue culture, similar to endothelial cells from human umbilical cord vein *in vitro* (28). Indirect immunofluorescence and $NaDodSO_4$/polyacrylamide gel electrophoresis studies showed that fibronectin is a major component of this matrix. The extracellular matrix described here appears to be morphologically different from the substrate-attached material found in fibroblast cultures (33), although this material does contain fibronectin (34).

Several lines of evidence suggest that the role of fibronectin in endothelial extracellular matrix may be to maintain a strong attachment of endothelial cells to the underlying basement membrane or extracellular matrix. First, addition of purified fibronectin to various transformed cell lines produces morphological alterations, such as cell flattening and a more untransformed appearance (15, 16), suggesting a role for fibronectin in cell–substrate adhesion. The ability of fibronectin to induce cell flattening may explain the extremely flattened appearance of cultured endothelial cells. Second, the appearance of fibronectin on cells in culture is dependent on the cell cycle (17) and is present in smaller amounts during or near division, when cells are less adherent to their substrate. Third, the appearance of fibronectin on cells in culture is dependent on cell–cell contact (18). Fourth, fibronectin mediates the adhesion of fibroblasts in culture to collagen (19, 20). Since fibronectin binds strongly to collagen and can be purified on collagen affinity columns (32), it is possible that fibronectin mediates the adhesion of endothelial cells to the extracellular matrix by interacting with collagen in the extracellular matrix or basement membrane *in vivo*. Considering that the vascular endothelium is subject to the severe hydrodynamic forces of blood, it is important that vascular endothelial cells are rigidly anchored to the underlying basement membrane to prevent the interaction of the basement membrane with blood components.

We found that most of the fibronectin in ABAE cell cultures exists in the extracellular matrix once the cells reach confluence and that very little fibronectin appears to be on top of the cell monolayer, at least as detected by immunofluorescence. These findings agree with those of Linder *et al.* (14), who used immunofluorescence staining of frozen and fixed sections to show that fibronectin is associated with the basement membrane *in vivo* and not with the overlying endothelial cells in large arteries. Immunofluorescence studies on cultured fibroblasts showed that fibronectin appears underneath the cells (35, 36), but fibronectin also appears in a fibrillar network over the cell surface (18, 30, 35, 36). Thus, endothelial cells may be unique in expressing fibronectin at confluence only in the extracellular matrix underneath the cell monolayer, but not on top of the monolayer.

At present no major physiological role has been ascribed to fibronectin, although recent evidence suggests that this protein is probably involved in cell–cell and cell–substrate adhesion (15–18). For vascular endothelial cells, our results also suggest that fibronectin is involved in cell–substrate adhesion and, furthermore, the large amount of fibronectin in the extracellular matrix of these cells may be responsible for their flattened and contact-inhibited morphology. Because the vascular endothelium is directly exposed to the blood, the endothelium may be a major source of cold-insoluble globulin, the plasma form of fibronectin that is probably shed from cells into the blood.

These studies were supported by National Cancer Institute Contract NO1-CB-74153 from the Tumor Immunology Program, U.S. Public Health Service Grant RO1-CA-15122, and American Cancer Society Grant BC-211 to G.L.N. C.R.B. was supported by National Institutes of Health Postdoctoral Fellowship 1 F32 CA05653-01.

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