SYNTHESIS OF FIBRONECTIN
BY CULTURED HUMAN ENDOTHELIAL CELLS

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Fibronectin (FN), a glycoprotein composed of high-molecular-weight polypeptide subunits, is present in substantial amounts in the plasma of vertebrates. It was first identified in 1948 and was previously referred to as cold-insoluble globulin (CIg). In this paper, the FN species found in plasma that contain a 200,000-dalton polypeptide subunit will be designated "plasma FN." In the last several years, an immunologically similar protein with a 220,000-dalton polypeptide subunit has been found in the postculture medium of fibroblasts; the same antigen has been identified by immunofluorescence microscopy in vertebrate basement membrane and loose connective tissue. This FN species, which we shall call "cellular FN," is also known as surface fibroblast antigen (SFA), large, external, transformation-sensitive (LETS) protein, and cell surface protein (CSP). Cellular FN is one of the major proteins synthesized by cultured fibroblasts and astroglial cells and is also found in myoblasts and some epithelial cells. Fibronectin in fibroblasts is found both in a soluble form in conditioned medium and in insoluble forms in the extracellular connective tissue matrix and on the cell surface. The insoluble forms may be spatially related to collagen: when fibroblast cultures are studied by immunofluorescence microscopy, the same filamentous extracellular structures are labeled by both FN and collagen antibodies. Plasma FN has recently been shown to bind to collagen and gelatin. Plasma FN is probably the protein in serum-containing tissue culture medium that mediates initial cell attachment and spreading on the surfaces of culture vessels. The cellular FN synthesized and deposited underneath cells may mediate adhesion of cells to culture dishes and other, more biologically relevant surfaces. Transformed cultured fibroblasts generally lack cell surface FN reviewed in Reference 21. When purified cellular FN is added to transformed cells, the transformed cells assume a more normal morphology and state of adhesiveness. The relationship between plasma and cellular FN is not clear. Both the insoluble and the soluble FN synthesized by cultured fibroblasts have the same-sized polypeptide subunit which has a molecular weight of 220,000 daltons. While it is hypothesized that plasma FN is a breakdown product of cellular FN, fibroblast cell surface FN appears to have a slightly larger polypeptide subunit than does plasma FN. Thus, the site of synthesis of plasma FN may not be any of those cells previously shown to synthesize cellular FN.

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We and others have previously shown that cultured human endothelial cells synthesize extracellular material that is morphologically and immunologically like amorphous basement membrane and biochemically like basement membrane collagen. Cultured endothelial cells also appear to synthesize extracellular material morphologically similar to microfibrils and elastic fibers. Recent immunofluorescence microscopic studies have demonstrated FN in the subendothelium of vessels in developing chicks and in human tissues. In this paper, we report that cultured human endothelial cells synthesize FN with a polypeptide subunit molecular weight of 200,000. Cultured endothelial cells secrete FN into culture medium and incorporate FN into the extracellular matrix. We suggest that endothelial cells may be a major site of synthesis of plasma FN.

**Materials and Methods**

**Cell Culture Techniques and Culture Media**

Human endothelial cells were derived from umbilical cords and cultured by use of methods and materials described previously. Endothelial cells were cultured in plastic T-25 or T-75 flasks (Corning). The flasks were pretreated by incubation overnight at 37°C with 0.2% gelatin (Difco) in water. The gelatin was removed just before usage. The culture medium consisted of medium 199 (Flow Laboratories) that contained either 20% fetal calf serum (Reheis Chemical Company) or 20% normal rabbit serum (Pel-Freez Biologicals). The culture media also contained penicillin (100 U/ml), streptomycin (100 μg/ml), l-glutamine (2 mM), and Hepes buffer (15 mM, pH 7.4, Sigma Chemical Company).

**Antibodies and Antigens**

Plasma FN and goat and rabbit antibodies to human plasma FN (designed anti-FN) were prepared as described previously. The unabsorbed rabbit anti-FN had no detectable antifactor VIII procoagulant activity or antivon Willebrand factor activity when assayed by methods described previously. However, due to the possibility of small amounts of contaminating factor-VIII antigen in the material used to elicit the anti-FN sera, all of the anti-FN sera used in this study were absorbed with purified plasma factor VIII before use. Anti-FN sera were absorbed by reacting 4 vol of anti-FN with 1 vol of purified factor VIII (0.98 mg/ml) at 37°C for 1 hr and incubating overnight at 4°C. The anti-FN sera were then centrifuged at 8000g for 20 min at 4°C. When the absorbed anti-FN sera and a well-characterized antifactor-VIII serum were reacted by immunodiffusion against a mixture that contained FN and factor VIII, lines of non-identity were seen, and antifactor VIII also detected free factor VIII antigen in the anti-FN sera.

**Radioactively Labeled Fibronectin Synthesized by Endothelial Cells**

Radioactively labeled FN was prepared by culturing monolayers of human endothelial cells in T-75 flasks in leucine-free MEM that contained 10% heat-inactivated rabbit serum, L-[4,5-3H]leucine (20 μCi/ml; sp act, 50 Ci/mmol;
Amesham/Searle Corporation), and Trasylo1® (50 U/ml, FBA Pharmaceuticals) and incubating the cells for 24 hr at 37°C. The postculture medium was removed, and the following inhibitors were added to it to yield the listed final concentration: 0.4 mM phenylmethylsulfonyl fluoride (Sigma Chemical Company), 5 mM N-ethylmaleimide (Sigma), 10 mM EDTA, and 1 μM pepstatin (Protein Research Foundation, Osaka, Japan). The postculture medium was precipitated with 50% saturated ammonium sulfate; the precipitate was then redissolved in phosphate-buffered saline (PBS, 1/10 original volume) that contained the four inhibitors listed above, dialyzed at 4°C against two changes of 2 liters each of PBS, and stored frozen at −40°C. This procedure yielded approximately 2 ml of a protein solution that contained 18.6 mg/ml of protein and 4.54 × 10^7 dpm/ml (sp act. 2.44 × 10^6 dpm/mg).

**Immunoisolation of [3H]Fibronectin**

Fibronectin synthesized by cultured endothelial cells was isolated from fractions derived from the postculture medium by a method that employs anti-FN coupled to a protein A-Sepharose column. The method is a modification of that of Kessler.13 To inhibit proteolytic enzymes, phenylmethylsulfonyl fluoride (0.4 mM), EDTA (10 mM), N-ethylmaleimide (5 mM), and pepstatin (1 μM) were added to all buffer and protein solutions used in these procedures.

Protein A bound to Sepharose 4B (Pharmacia Fine Chemicals) was washed twice with 0.15 M NaCl, 0.02 M Tris HCl (pH 7.4). Two-tenths of a milliliter of the washed beads, 0.2 ml of Tris-buffered saline (TBS), and 0.15 ml of anti-FN were incubated with end-over-end rotation for 2 hr at 20°C. To saturate the remaining protein A-binding sites, 2 ml of heat-inactivated normal rabbit serum were added, the mixture was incubated for 1 hr at 4°C, and the beads were then washed six times with PBS. The washed beads were suspended in 0.75 ml of PBS, 0.25 ml of the [3H]-labeled ammonium sulfate-precipitated postculture medium was added, and the mixture was incubated overnight at 4°C. The suspension was transferred to a 0.6 × 15 cm chromatographic column (Glenco Scientific Inc.), and the excess fluid was drained off. The beads (0.2 ml packed volume) were washed sequentially with PBS (6 ml), 5 M KI (8 ml), 1% Triton® X-100 (Sigma) in PBS (8 ml), and PBS (5 ml). The beads were then eluted with 1 M acetic acid, and the eluate was analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. The washes with 5 M KI and 1% Triton X-100 were added because in preliminary experiments, FN was noted to adhere nonspecifically to protein A-Sepharose. Kessler32 has shown that 5 M KI and the nonionic detergent NP-40 do not release appreciable amounts of antigen-antibody complexes bound to protein A. We found that washes with 5 M KI and 1% Triton X-100 in PBS release nonspecifically bound FN but do not release FN bound to anti-FN. Control experiments were performed with antiovalbumin and antihuman serum albumin.

**Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis**

This procedure was performed in 3% polyacrylamide-0.5% agarose gels by the method of Weinstein et al.14 Samples in 1 M acetic acid were prepared for electrophoresis by heating in sample buffer for 1 hr at 37°C. The samples were then dialyzed against running buffer that contained 1% SDS, 0.4 mM phenylmethyl-
sulfonyl fluoride, 5 mM N-ethylmaleimide, 10 mM EDTA, and 1 \( \mu \)M pepstatin for 2 hr at 20°C. Samples to be reduced were treated with 3 \( \mu l/100 \mu l \) of a 1.4 M dithiothreitol solution by boiling for 5 min. Gels were sectioned into 2-mm thick slices, processed for liquid scintillation counting as described previously,\(^{32}\) and counted in a Searle Mark III liquid scintillation counter. Purified human plasma factor VIII (subunit mol wt, 202,000) used as a marker was prepared as described previously.\(^{32}\) Purified plasma FN used as a marker (subunit mol wt, 200,000) was purified as described previously.\(^{2}\)

The 3% polyacrylamide-0.5% agarose system was chosen because when samples are run unreduced in this system, factor VIII and plasma FN are completely separated from each other, whereas with reduction, they have the same mobility.\(^{34}\)

**FIGURE 1.** Immunofluorescence study of cultured human endothelial cells. Cells were treated with anti-FN and then with fluorescein-conjugated goat antirabbit IgG. A meshwork of extracellular fibrils is brightly stained. x960.

**RESULTS**

**Immunofluorescence Studies**

When coverslips of confluent cultures of human endothelial cells, cultured in normal rabbit serum and fixed in acetone, were incubated sequentially with rabbit anti-FN and fluorescein-conjugated goat antirabbit IgG, meshworks of extracellular fibrils were brightly stained (**FIGURE 1**). When subconfluent endothelial cells were similarly stained, only rare immunofluorescent extracellular fibrils were seen, although large numbers of brightly fluorescent intracellular granules were present. As the cells became more confluent, the numbers of intracellular im-
munofluorescent granules decreased, and the extent of the extracellular immunofluorescent fibrillar meshwork increased. By the time the cells were well confluent, only rare immunofluorescent intracellular granules were seen. The fibrils were not related to areas of cell-cell junctions; instead, they appeared to form a continuous meshwork underlying the cells. No staining was evident when the cultured endothelial cells were stained with normal rabbit serum, antiprothrombin, or antiserum to antithrombin III instead of anti-FN.

Immunofluorescence staining of endothelial cells was completely inhibited by prior absorption at equivalence of anti-FN serum by purified plasma FN. Supernatants from the zone of antigen excess did not stain cultured endothelial cells, whereas supernatants in the zone of antibody excess did.

**Endothelial Cell Fibronectin**

For this study, endothelial cells were cultured in media that contained 20% fetal calf serum, because the goat anti-FN was unable to detect FN in fetal calf serum by immunodiffusion analysis. Pre- and postculture media were concentrated fivefold and assayed for FN by radial immunodiffusion by use of goat anti-FN. Concentrated preculture media contained no detectable FN (Table I). Concentrated postculture media, however, did contain significant amounts of FN.

**Immunodiffusion Studies**

On immunodiffusion analysis, goat anti-FN reacted with a line of identity when tested against endothelial cell postculture medium and human plasma. Fetal calf serum, which was present in the culture medium utilized for these studies, did not form a precipitin line when tested against the goat anti-FN serum.

**Immunoisolation of Endothelial Cell Fibronectin**

Fibronectin synthesized by endothelial cells was isolated from fractions of postculture media derived from endothelial cells cultured in 20% normal rabbit serum.

**Table 1**

| Material Tested† | Fibronectin† (μg/culture flask) |
|------------------|---------------------------------|
| Preculture media | <8                              |
| Postculture media|                                 |
| Culture 1        | 50                              |
| 2                | 25                              |
| 3                | 55                              |
| 4                | 77                              |

*From Jaffe & Mosher.42
†Four separate strains of endothelial cells were cultured for 3 days, and the media were concentrated fivefold and analyzed by radial immunodiffusion.
¶The minimal amount of FN detectable by this technique was 8 μg per culture flask. The values represent averages of two sets of analyses on multiple dilutions of each sample.
or fetal calf serum with \(^{3}H\)leucine in the presence of Trasylol (50 U/ml). The \(^{3}H\)FN was isolated by two different methods; a technique that employs protein A-Sepharose and double-antibody immunoprecipitation. The protein A-Sepharose technique utilized a rabbit anti-FN serum to avoid interspecies cross-reactivity. The isolated \(^{3}H\)FN was characterized by SDS polyacrylamide gel electrophoresis with and without reduction by dithiothreitol.

A fraction obtained from \(^{3}H\)leucine-labeled endothelial cell postculture medium by precipitation with ammonium sulfate at 50% saturation was electrophoresed, sliced, and counted for radioactivity (Figure 2, A). This gel contained three major radioactive peaks, one of which comigrated with the human plasma FN used as a marker. The radioactively labeled ammonium sulfate-precipitated postculture medium was subjected to immunoisolation by use of anti-FN coupled to protein A-Sepharose. After this procedure, analysis of the depleted labeled postculture media by SDS polyacrylamide gel electrophoresis revealed that the radioactive peak that comigrated with the plasma FN marker (Figure 2, A) had almost completely disappeared (data not shown). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (after reduction) of the eluate from the anti-FN
protein A-Sepharose column (FIGURE 2, B), revealed one radioactive band with a molecular weight of 200,000 that comigrated with the marker plasma FN. When the acetic acid eluate was electrophoresed without reduction (data not shown), one radioactive band was seen, and it comigrated with the unreduced plasma FN marker. Control studies performed with antihuman serum albumin and antiovalbumin instead of anti-FN did not isolate any labeled material (FIGURE 2, B). In this gel system (3%, polyacrylamide-0.5% agarose), after reduction, the plasma factor-VIII marker comigrated with the plasma FN marker. However, when factor VIII was electrophoresed without reduction, it did not enter the gel, whereas the plasma FN marker did. Thus, this system could be used to completely separate FN and factor VIII.34

Labeling patterns identical to those shown in FIGURE 2 were seen when the radioactively labeled ammonium sulfate-precipitated postculture medium was subjected to double-antibody immunoprecipitation with anti-FN and goat anti-rabbit IgG. Control double-antibody immunoprecipitation experiments with antihuman serum albumin and antiovalbumin instead of anti-FN precipitated no labeled material.

**DISCUSSION**

The studies reported here demonstrate that cultured human endothelial cells synthesize and release FN into culture media and incorporate FN into the extracellular matrix. The radioactive FN secreted by endothelial cells into the culture medium comigrated with the plasma FN marker on SDS polyacrylamide gel electrophoresis both with and without reduction and had a single polypeptide subunit with a molecular weight of 200,000 daltons, similar to the plasma FN marker. Unlike endothelial cell FN, the FN synthesized by some fibroblasts has a subunit molecular weight of 220,000 in both its soluble and insoluble (cell surface-bound and extracellular matrix-embedded) forms.4,6,24 Since the fibronectin synthesized by fibroblasts consists of 220,000-dalton subunit molecular weight polypeptides, which are apparently not transformed into the polypeptides of 200,000 molecular weight found in plasma,24 endothelial cells may well synthesize the bulk of plasma fibronectin that consists of polypeptides of 200,000 molecular weight.

The validity of these studies is dependent on the specificity of the anti-FN sera used. These antibodies have previously been shown to form a single line of identity when tested against human plasma by immunodiffusion analysis and immunoelectrophoresis.5,28 The quantitative immunoprecipitin curve and the loss of immunofluorescence at equivalence (FIGURE 2) also argue strongly for monospecificity. We have previously shown that cultured endothelial cells contain, synthesize, and release factor-VIII antigen.30,32,35 To avoid the possibility of contamination of the anti-FN sera with trace amounts of antifactor VIII, all anti-FN sera were absorbed with purified plasma factor VIII before use. These absorptions and the pattern of mobility of the [3H]FN peak seen in FIGURE 2 and that obtained on electrophoresis without reduction rule out the possibility that the isolated radioactive peak seen in FIGURE 2 is factor-VIII antigen.

Fibronectin is one of the major proteins synthesized and secreted by cultured endothelial cells. It appears to constitute approximately 15% of the protein released by the cells into the culture media. By comparison, factor-VIII antigen represented only 5.5% of a high-molecular-weight fraction (separated by Sepharose 6B) prepared from the same starting material35 and thus probably repre-
sented less than 1% of the total protein released by the cells into the culture medium. These estimates are consistent with the concentrations of these proteins in plasma, namely, factor VIII antigen, approximately 5–10 μg/ml, and plasma FN, about 330 μg/ml. These estimates are also consistent with the results obtained when anti-FN bound to protein A-Sepharose was reacted with the radioactively labeled, ammonium sulfate-precipitated postculture medium. Practically the entire 200,000-dalton peak seen in Figure 2 (A) was removed; the remaining radioactivity in the peak area (~5% of the initial amount) probably represents factor-VIII antigen. Cultures of human endothelial cells secreted an average of 51.8 μg of FN/25 cm² of dish during 3 days in culture (Table 1). By comparison, a variety of first-passage human embryonic cell strains secreted an average of 106 μg of FN/25 cm² of dish (equivalent) during 3 days in culture.

Fibronectin has been shown to be present in the subendothelium of a variety of blood vessels. Linder et al., in studies on the developing chick embryo, showed that in addition to loose connective tissue, the subendothelium of arteries and veins and also of brain capillaries contained FN when tissue sections were studied by immunofluorescence microscopy. Fibronectin was absent in parenchymal cells in a variety of organs, such as liver, striated and smooth muscle, brain (except neural sheaths), cartilage, and bone. Similar studies on sections of human thyroid have shown that FN is present in the basement membrane that surrounds the colloid follicles and in the subendothelium of blood vessels.

The physiologic role of FN is not clear, although several of its effects are known. Fibronectin is necessary for the attachment of tissue culture cells to culture vessels. Fibronectin increases the strength of attachment of transformed cells to culture vessels and thus changes their morphology (but not their growth characteristics and malignant potential). The mechanism(s) responsible for these two effects is unknown. Fibronectin also seems to be an important constituent of basement membranes and loose connective tissue, where it forms extensive meshworks of extracellular fibrils. The same fibrils have also been shown to contain collagen when examined by immunofluorescence microscopy. The basis for this congruence of location may be the recently described noncovalent interactions of FN with other FN molecules and with collagen and gelatin. In addition, FN cross-links with itself and other proteins through disulfide bonds. Fibronectin on cell surfaces is known to be immobile and thus may serve as an anchorage point for cell attachment. Fibronectin can be covalently cross-linked by factor XIII both to other FN molecules and to the α-chain of fibrin. This interaction presumably allows fibrin in wounds to attach to fibroblasts. Fibroblasts are known to be able to contract fibrin clots in vitro; in vivo, these reactions are probably important in wound healing. Muir et al. have suggested that FN may be similar to a microfibrillar protein isolated from cultured monkey smooth muscle cell extracellular matrix. We suggest an additional possibility: that FN in the extracellular matrix is the high-molecular-weight glycoprotein (subunit polypeptide molecular weight after reduction, ~200,000 daltons) isolated by Kefalides from collagenase-digested glomerular basement membranes. Thus, endothelial cell fibronectin may be an important component of the subendothelium that influences normal blood vessel physiology.

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