SYNTHESIS OF FIBRONECTIN BY CULTURED HUMAN ENDOTHELIAL CELLS*

By ERIC A. JAFFE‡ and DEANE F. MOSHER

(From the Division of Hematology-Oncology, Department of Medicine, Cornell University Medical College, New York 10021; and the Division of Hematology, Department of Medicine, University of Wisconsin Medical School, Madison, Wisconsin 53706)

Plasma fibronectin (FN)[1] is probably the major nonimmune particulate opsonin of blood (1, 2). FN is also cross-linked to fibrin during the final stage of blood coagulation (3, 4). It was first identified in 1948 and was previously referred to as cold insoluble globulin (5). FN is a glycoprotein composed of high molecular weight polypeptide subunits which is present in human plasma in concentrations of 300–400 μg/ml (3, 6). In the last several years, an immunologically similar protein with a 200–200,000 mol wt 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 (7–9). This antigen is also known as surface fibroblast antigen (7), large, external, transformation-sensitive protein (10), and cell-surface protein (11). Cultured fibroblasts (12–15) and astroglial cells (16) have previously been identified as sites of syntheis of FN. FN in fibroblast cultures 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 for when fibroblast cultures are studied by immunofluorescence microscopy, the same filamentous extracellular structures are labeled by both FN and collagen antibodies (17). Also, FN has recently been shown to bind to collagen (18). FN is probably the protein in serum-containing tissue culture medium which mediates initial cell attachment and spreading on the surfaces of culture vessels (19–21). FN synthesized and deposited underneath cells may mediate adhesion of cells to culture dishes and other, more biologically relevant surfaces (22). Transformed cultured fibroblasts generally lack cell surface FN (10, 11, 14–16, reviewed in 23). When purified FN is added to transformed cells, the transformed cells assume a more normal morphology and state of adhesiveness (24, 25).

We and others have previously shown that cultured human endothelial cells synthesize extracellular material which is morphologically and immunologically like amorphous basement and contains basement membrane collagen (26, 27). Cultured endothelial cells also appear to synthesize extracellular material morphologically similar to microfibrils and elastic fibers (26). Recent immunofluorescence microscopic studies have demonstrated FN in the endothelium and subendothelium of vessels in developing chicks (6) and in human tissues (28, 29).

* Supported by grants from the National Institutes of Health, HL-18828, the Arnold R. Krakower Hematology Foundation, and the University of Wisconsin Graduate School.

‡ Recipient of National Institutes of Health Research Career Development Award (1 K04 HL 00237) and a Career Scientist Award from the Irma T. Hirschl Trust.

[1] Abbreviations used in this paper: FN, fibronectin; MEM, minimal essential medium; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline.

In this paper, we report that cultured human endothelial cells synthesize FN with a polypeptide subunit mol wt 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 FN.

Materials and Methods

Cell Culture Techniques and Culture Media. Human endothelial cells were derived from umbilical cords and cultured by using methods and materials previously described (30). Endothelial cells were cultured in plastic T-25 or T-75 flasks (Corning Glass Works, Science Products Div., Corning, N. Y.). The flasks were pretreated by incubation overnight at 4°C with 0.2% gelatin (Difco Laboratories, Detroit, Mich.) in water. The gelatin was removed just before use. Coverslips on which cells were cultured for immunofluorescence microscopy were similarly treated with gelatin. The culture medium for endothelial cells consisted of medium 199 (Flow Laboratories, Inc., Rockville, Md.) containing either 20% fetal calf serum (Reheis Chemical Co., Chicago, Ill.) or 20% normal rabbit serum (Pel-Freez Farms Inc., Rogers, Ark.). The culture media also contained penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM) (all Flow Laboratories) and Hepes buffer (15 mM, pH 7.4, Sigma Chemical Co., St. Louis, Mo.).

Immunofluorescence Microscopy. 2–7 days after subculture, cultured cells were prepared and processed for immunofluorescence microscopy by previously described methods (30, 31). The goat anti-rabbit IgG which was purified by affinity chromatography and conjugated with fluorescein according to methods previously described (32) was kindly provided by Dr. Carl G. Becker (Cornell University Medical College, New York). The various rabbit antibodies and normal rabbit serum were used at dilutions of 1:10 or 1:20 and 20 μl of antiserum were used per coverslip. Immunofluorescence microscopy and photomicrography were performed by methods previously described (30).

Antibodies and Antigens. Plasma FN and goat and rabbit antibodies to human plasma FN (designed anti-FN) were prepared as previously described (3, 4). The unabsorbed rabbit anti-FN had no detectable anti-factor VIII procoagulant activity or anti-von Willebrand factor activity when assayed by methods previously described (33, 34). However, due to the possibility of small amounts of contaminating factor VIII antigen in the material used to elicit the anti-FN sera, all the anti-FN sera used in this study were absorbed with purified plasma factor VIII (35) 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 h and overnight at 4°C. The anti-FN sera were then centrifuged at 8,000 *g* for 20 min at 4°C. When the absorbed anti-FN sera and a well characterized anti-factor VIII serum (35) were reacted in immunodiffusion against a mixture containing FN and factor VIII, lines of nonidentity were seen and the anti-factor VIII also detected free factor VIII antigen in the anti-FN sera. When anti-factor VIII and absorbed anti-FN sera were tested by immunofluorescence microscopy with confluent cultured endothelial cells, the antisera gave different patterns of fluorescence. The rabbit anti-FN stained mainly an extensive meshwork of extracellular fibrils while the anti-factor VIII stained only intracellular granules.

The antisera to human serum albumin was obtained from Behring Diagnostics, American Hoechst Corp., Somerville, N. J. The antisera to ovalbumin was kindly provided by Dr. Gordon D. Ross, Cornell University Medical College, New York. The antisera to anti-thrombin III and prothrombin were provided by Dr. Robert Rosenberg, Harvard Medical School, Boston, Mass. All antisera were absorbed with aluminum hydroxide and heat-inactivated before use.

Quantitative Absorption Studies. Rabbit anti-FN was diluted 1:4 with phosphate-buffered saline ([PBS] 0.145 M NaCl, 0.01 M phosphate, pH 7.4) and 0.2-ml portions pipetted into a series of Eppendorff centrifuge tubes. To these were added serial twofold dilutions (0.2 ml) of purified human FN, the highest concentration being 488 μg/tube. The tubes were incubated with frequent shaking at 37°C for 2 h and then at 4°C for 72 h. The precipitate harvested by centrifugation was washed four times in PBS, dissolved in 1 N NaOH, and protein content determined by the Lowry et al. procedure (36). Portions of each supernate were used to treat cultured endothelial cells as described above to determine if absorption of anti-FN serum at equivalence with purified FN would inhibit immunofluorescence staining. These preparations were compared with cultured endothelial cells treated with unabsorbed anti-FN at the same final dilution (1:20) as the

supernate and with cells treated with pooled normal rabbit serum. Using the data obtained from the quantitative immunoprecipitation study (Fig. 2), we calculated that the anti-FN serum contained 2.46 mg/ml of specific anti-FN antibody.

Radial Immunodiffusion. Radial immunodiffusion was performed by the method of Mancini et al. (37) with goat anti-FN at a final dilution of 1:140. Confluent endothelial cells were cultured for 3 days in T-75 flasks in 10 ml of culture media containing 20% fetal calf serum. The postculture media was removed, centrifuged to remove debris, lyophilized, reconstituted in 1/5 the original volume of PBS, dialyzed against PBS, and analyzed. Human plasma of known FN concentration served as a secondary standard. The primary standard was purified plasma FN; protein concentration of the primary standard was determined by absorbance at 280 nm (6). The minimal level of FN detectable by this technique in a 10-μl sample was 40 ng. Thus, the minimal level detectable in the culture media was 8 μg/flask (10 ml of culture media). The goat anti-FN sera used in this study did not detect FN in preculture media or in the fetal calf serum used in the culture medium.

Immunodiffusion analysis. Immunodiffusion analysis was performed on glass slides containing 1% agarose in 50 mM Tris-barbital buffer pH 8.2. Samples to be tested (10 μl) were placed in 4-mm diameter wells and the plates incubated at room temperature for 48 h. The anti-FN was used at a dilution of 1:2. The plates were washed extensively with Tris-buffered saline and then water, stained with Coomassie Brilliant Blue, and photographed.

Radioactively Labeled FN Synthesized by Endothelial Cells. Radioactively labeled FN synthesized by endothelial cells was prepared by culturing monolayers of human endothelial cells in T-75 flasks in medium 199 containing 20% normal rabbit serum until the cells were confluent. The medium was then removed and the cells washed three times with Dulbecco's PBS. The cells were incubated in leucine-free minimal essential medium (MEM) (Flow Laboratories) containing 20% heat inactivated rabbit serum for 30 min at 37°C. This medium was removed and replaced with 10 ml of fresh leucine-free MEM containing 20% heat inactivated rabbit serum, L-[4, 5-^{3}H] leucine (20 μCi/mol, sp act 50 Ci/mmol, Amersham Corp., Arlington Heights, Ill.), and Trasylol (50 U/ml, FBA Pharmaceuticals, Inc., New York) and the cells incubated for 24 h at 37°C. The postculture medium was removed and the following inhibitors added to it to yield the listed final concentration: 0.4 mM phenyl methyl sulfonyl fluoride (Sigma Chemical Co.), 5 mM *N*-ethyl maleimide (Sigma Chemical Co.), 10 mM EDTA, and 1 μM pepstatin (Protein Research Foundation, Osaka, Japan). The postculture medium was centrifuged at 40,000 *g* for 1 h at 4°C to sediment particulate debris. The supernate was made up to 50% saturation with ammonium sulfate, incubated at 4°C overnight, and centrifuged at 40,000 *g* for 1 h at 4°C. The precipitate from 2 T-75 flasks was redissolved in PBS (1/10 original volume) containing 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 containing 18.6 mg/ml protein and 4.54×10^7 dpm/ml (sp act 2.44×10^6 dpm/mg).

The [^{3}H]-labeled endothelial cell monolayers were washed four times with medium 199 containing 10^{-4} M leucine and once with medium 199 containing 10^{-4} M leucine and 0.4 mM phenyl methyl sulfonyl fluoride. The cell monolayers were then extracted for 2 h at 37°C with medium 199 containing 1 M urea and 0.4 mM phenyl methyl sulfonyl fluoride (11, 38). The extract was removed and *N*-ethyl maleimide, EDTA, and pepstatin added to yield the same final concentrations as used in processing the postculture medium. The urea extract was centrifuged at 40,000 *g* for 15 min and then dialyzed against PBS at 4°C. This procedure yielded approximately 6.5 ml of a solution containing 1.75×10^6 dpm/ml.

Immunodiffusion Analysis. Immunodiffusion analysis was performed on glass slides containby cultured endothelial cells was isolated from fractions derived from postculture medium and from urea extracts of cellular monolayers by a method using anti-FN. The method used a Protein A-Sepharose column and is a modification of the method of Kessler (39). To inhibit proteolytic enzymes, phenyl methyl sulfonyl fluoride (0.4 mM), EDTA (10 mM), *N*-ethyl maleimide (5 mM), and pepstatin (1 μM) were added to all buffer and protein solutions used in these procedures.

Protein A-Sepharose 4B (Protein A bound to Sepharose 4B, Pharmacia Fine Chemicals, Piscataway, N. J.) was washed twice with 0.15 M NaCl, 0.02 M Tris-HCl, pH 7.4 (TBS-Tris-buffered saline). 0.2 ml of the washed beads, 0.2 ml TBS, and 0.15 ml of the anti-FN were incubated with end-over-end rotation for 2 h at 20°C. To saturate the remaining Protein A binding sites, 2 ml of heat-inactivated normal rabbit serum was added, the mixture incubated for 1 h at

4°C, and the beads then washed six times with PBS. The washed beads were suspended in 0.75 ml of PBS, 0.25 ml of the [^{3}H]-labeled ammonium sulfate precipitated postculture meidum or 0.9 ml of the dialyzed cell extract was added, and the mixture incubated overnight at 4°C. The suspension was transferred to a 0.6 × 15 cm chromatographic column (Glenco Scientific Inc., Houston, Tex.) and the excess fluid 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 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 stick nonspecifically to Protein A-Sepharose. Kessler (39) 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 did not release FN bound to anti-FN. Control experiments were performed with anti-ovalbumin and anti-human serum albumin.

Immunoisolation of [^{3}H] Endothelial Cell FN by Double Antibody Immunoprecipitation. Double antibody immunoprecipitation reactions were carried out on [^{3}H]leucine-labeled ammonium sulfate precipitated endothelial cell postculture medium prepared as above except that the endothelial cells were labeled while in fetal calf serum. The cells were thus cultured to avoid adding large amounts of rabbit gamma globulins which would have interfered with the second immunoprecipitation step (goat anti-rabbit IgG precipitating the rabbit anti-FN). 38 μl of rabbit anti-FN was added to 0.5 ml [^{3}H]-labeled ammonium sulfate precipitated postculture medium and the mixture incubated for 2 h at 37°C and overnight at 4°C. Goat anti-rabbit IgG (0.2 ml) was added and the mixture further incubated for 2 h at 37°C and for 2 days at 4°C. The mixture was centrifuged at 8000 *g* for 5 min at 4°C. The supernate was removed and saved for analysis and the precipitate washed six times by centrifugation with PBS containing 0.5% Triton X-100 (39). The supernates and pellets were then analyzed by SDS-polyacrylamide gel electrophoresis. Control experiments were performed with anti-ovalbumin and anti-human serum albumin. Preliminary quantitative immunoprecipitation experiments were performed to determine the equivalence point for the reaction between the goat anti-rabbit IgG and the various antibodies.

SDS-Polyacrylamide Gel Electrophoresis. SDS-polyacrylamide gel electrophoresis was performed in 3% acrylamide-0.5% agarose gels by the method of Weinstein et al. (40). Samples in 1 M acetic acid were prepared for electrophoresis by heating in sample buffer for 1 h at 37°C. The samples were then dialyzed against running buffer containing 1% SDS, 0.4 mM phenyl methyl sulfonyl fluoride, 5 mM *N*-ethyl maleimide, 10 mM EDTA, and 1 μM pepstatin for 2 h at 20°C. Samples to be reduced were treated with 3 μl/100 μl of a 1.4 M dithiothreitol solution by boiling for 5 min. Immunoprecipitates and samples not in acetic acid were solubilized by boiling for 5 min in sample buffer. Gels were sectioned into 2-mm thick slices and processed for liquid scintillation counting either as previously described (35) or by incubating the gel slices in 10 ml of 3% Protosol in Econofluor (New England Nuclear Corp., Boston, Mass.) for 16–24 h at 37°C (the two methods yielded the same results). The samples were counted in a Searle Mark III liquid scintillation counter (Searle Analytic Inc., Arlington Heights, Ill.). Purified human plasma factor VIII (subunit mol wt 202,000) used as a marker was prepared as previously described (35). Purified plasma FN used as a marker (subunit mol wt 200,000) was purified as previously described (3).

The 3% polyacrylamide-0.5% agarose system was chosen because in this system when samples are run unreduced, factor VIII and plasma FN are completely separated from each other whereas with reduction they have the same mobility (40).

Results

Immunofluorescence Studies. When coverslips of confluent cultures of human endothelial cells, cultured in normal rabbit serum and fixed in acetone, were sequentially incubated with rabbit anti-FN and fluorescein-conjugated goat anti-rabbit IgG, meshworks of extracellular fibrils covering all the cells were brightly stained (Fig. 1). When subconfluent endothelial cells were similarly stained, few immunofluorescent extracellular fibrils were seen though large numbers of brightly fluorescent intracellular granules were present. As

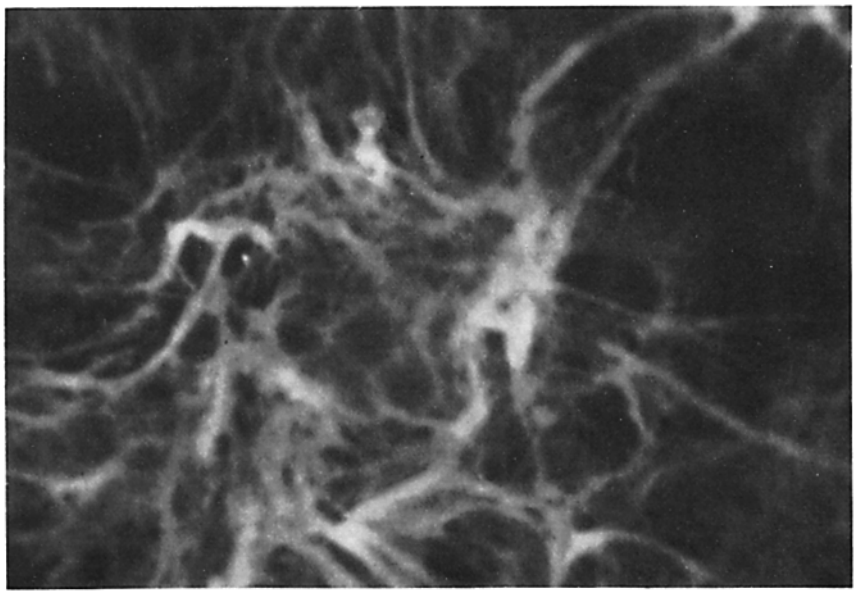

FIG. 1. Immunofluorescence study of cultured human endothelial cells cultured in rabbit serum. Cells were treated with rabbit anti-FN and then with fluorescein-conjugated goat anti-rabbit IgG. A meshwork of extracellular fibrils is brightly stained; the cells are very lightly stained and are barely visible because the extracellular matrix stains so brightly (× 960).

the cells became more confluent, the numbers of intracellular immunofluorescent granules decreased somewhat and the extent of the extracellular immunofluorescent fibrillar meshwork greatly increased. 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 seen when the cultured endothelial cells were stained with normal rabbit serum, anti-prothrombin, or antisera to anti-thrombin III instead of anti-FN. To demonstrate the endothelial origin and homogeneity of the cells, parallel coverslips were also stained for factor VIII antigen using an antibody to human factor VIII antigen (31, 35). All the cells were brightly stained thus confirming their endothelial origin (31).

Immunofluorescence staining of endothelial cells was completely inhibited by prior absorption at equivalence of anti-FN serum by purified FN (Fig. 2). Supernates from the zone of antigen excess did not stain cultured endothelial cells, whereas supernates in the zone of antibody excess did.

Endothelial Cell FN. For this study, endothelial cells were cultured in media containing 20% fetal calf serum since the goat anti-FN was unable to detect FN in fetal calf serum by immunodiffusion analysis. Pre- and postculture media were concentrated five-fold and assayed for FN by radial immunodiffusion using goat anti-FN. Concentrated preculture media contained no detectable FN (Table I). However, concentrated postculture media from four different endothelial cell strains 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

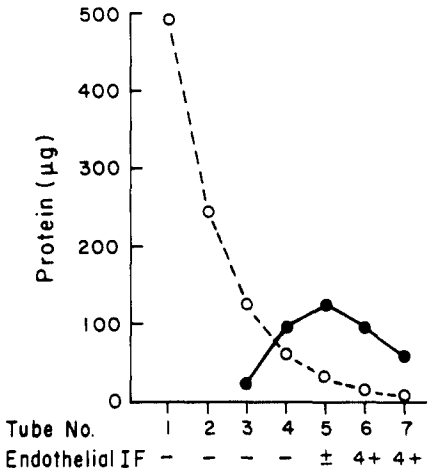

FIG. 2. Quantitative precipitin analysis of the FN-anti-FN reaction. Serial dilutions of purified human FN (-○-) were added to a constant amount of rabbit antiserum to FN. The resulting immunoprecipitates (-●-) were quantitated by the Lowry procedure. The ability of the supernates to stain cultured endothelial cells was tested by immunofluorescence as detailed in the Materials and Methods section.

TABLE I
FN Content of Pre and Postculture Media

Material tested*	FN‡
	µg/culture flask
Preculture media	<8
Postculture media	
Culture	
1	50
2	25
3	55
4	77

* Four different endothelial cell lines 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/culture flask. The values represent averages of two sets of analyses on multiple dilutions of each sample.

medium and human plasma (Fig. 3). Preculture medium, which contained fetal calf serum, did not form a precipitin line when tested against the goat anti-FN serum.

Immunoisolation of Endothelial Cell FN. FN synthesized by endothelial cells was isolated from fractions of postculture media derived from endothelial cells cultured in 20% normal rabbit or fetal calf serum with [^{3}H] leucine in the presence of Trasylol (50 U/ml). The [^{3}H] FN was isolated either by a technique using Protein A-Sepharose or by double antibody immunoprecipitation. The Protein A-Sepharose technique utilized a rabbit anti-FN sera and cells cultured and labeled in rabbit serum to avoid interspecies cross-reactivity. The isolated

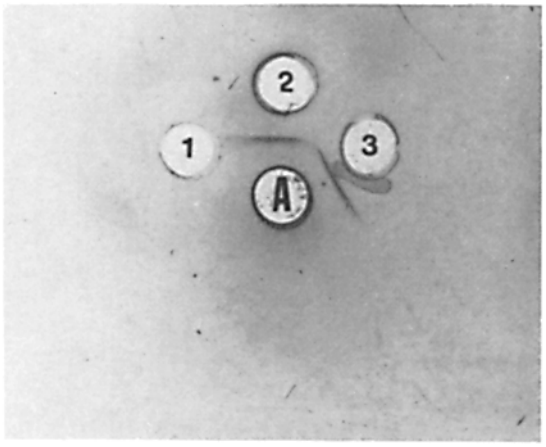

FIG. 3. Immunodiffusion analysis of FN synthesized by endothelial cells. The center well (A) contained goat anti-human FN. The numbered wells contained: 1) preculture medium; 2) human plasma; and 3) postculture medium. The stained immunodiffusion plate shows a line of identity between the FN in human plasma and the FN present in endothelial cell postculture medium. No FN was detected in the preculture medium.

[^{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 (Fig. 4 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 using anti-FN coupled to Protein A-Sepharose. After this procedure, analysis of the depleted labeled postculture media by SDS-polyacrylamide electrophoresis revealed that the radioactive peak comigrating with the plasma FN marker (Fig. 4 A) had almost completely disappeared (data not shown). SDS-gel electrophoresis (after reduction) of the acetic acid eluate from the anti-FN Protein A-Sepharose column (Fig. 4 B), revealed one radioactive band with a mol wt of 200,000 which comigrated with the plasma FN marker. When the acetic acid eluate was electrophoresed without reduction (Fig. 4 C), one radioactive band was seen and it comigrated with the unreduced plasma FN marker. Control studies performed with anti-human serum albumin and anti-ovalbumin instead of anti-FN did not isolate any labeled material (Figs. 4 B and 4 C). In this gel system (3% acrylamide-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 while the plasma FN marker did. Thus, this system could be used to completely separate FN and factor VIII (40).

Labeling patterns identical to those seen in Fig. 4 were seen when radioactively labeled ammonium sulfate precipitated postculture medium was subjected to double antibody immunoprecipitation with rabbit anti-FN and goat anti-rabbit IgG. Control double antibody immunoprecipitation experiments

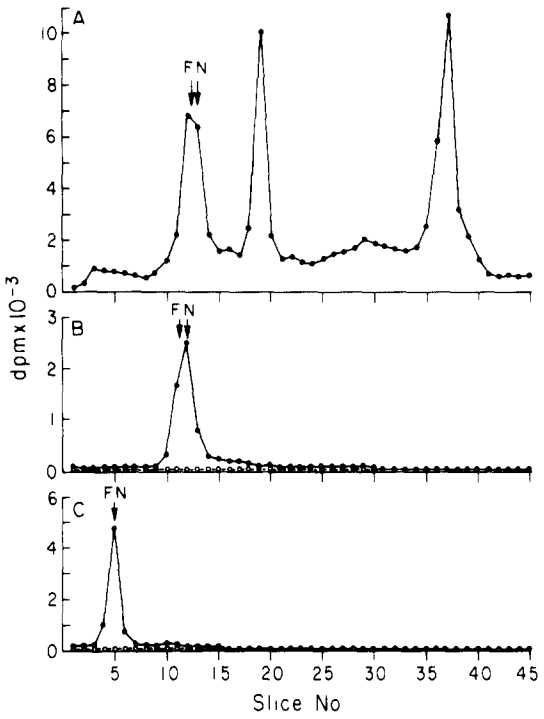

FIG. 4. Protein A-Sepharose isolation of FN synthesized by cultured endothelial cells. Distribution of radioactivity in SDS-polyacrylamide gels of: (A) fraction derived from [^{3}H] leucine-labeled endothelial cell postculture medium by precipitation with 50% saturated ammonium sulfate (reduced with dithiothreitol); (B) fraction derived by acetic acid elution of an anti-FN Protein A-Sepharose column which has been reacted with the [^{3}H] leucine labeled, ammonium sulfate precipitated endothelial cell postculture medium shown in (A) and then washed as detailed in the Materials and Methods Section (-●-●-). Control experiments were performed by using anti-human serum albumin and anti-ovalbumin (-○-○-) instead of anti-FN (all reduced with dithiothreitol); (C) same as (B) except unreduced by dithiothreitol. The arrows labeled FN represent the location of the plasma FN marker run on companion gels. When reduced, plasma FN electrophoreses as a closely spaced doublet with an average mol wt of 200,000, hence the double arrow.

using anti-human serum albumin and anti-ovalbumin instead of anti-FN precipitated no labeled material.

Since essentially all (≥95%) the radioactivity in the high molecular weight peak comigrating with plasma FN marker (Fig. 4 A) was FN and that peak represented ~15% of the output of [^{3}H] leucine-labeled protein by the endothelial cells, we estimate that FN represents ~15% of the protein released by endothelial cells into the culture medium.

Endothelial cell monolayers labeled with [^{3}H] leucine were extracted with 1 M urea for 2 h at 37°C since it has been shown that insoluble fibroblast FN can be extracted using this procedure (11, 38). The urea extract was analyzed by the Protein A-Sepharose technique described above. Labeling patterns identical to those seen in Fig. 4 B and 4 C were obtained. Control experiments performed with anti-ovalbumin instead of anti-FN did not isolate any 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 and incorporated into the extracellular matrix comigrated with the plasma FN marker on SDS-polyacrylamide gel electrophoresis both with and without reduction and had a single polypeptide subunit of 200,000 mol wt similar to the plasma FN marker. On immunodiffusion analysis, the FN secreted by cultured endothelial cells into their postculture medium formed a line of identity with human plasma when both were reacted against anti-FN. Thus, the FN synthesized and secreted by endothelial cells and plasma FN are immunologically identical and are both disulfide bonded dimers with subunits of the same molecular weight.

The validity of our studies is dependent on the specificity of the anti-FN sera used. These antibodies have previously been shown to form a single line when tested against human plasma by immunodiffusion analysis and immunoelectrophoresis (3, 4). The quantitive immunoprecipitin curve and the loss of immunofluorescence at equivalence (Fig. 2) also argue strongly for monospecificity. We have previously shown that cultured endothelial cells contain, synthesize, and release factor VIII antigen (31,33, 35). To avoid the possibility of contamination of the anti-FN with trace amounts of anti-factor VIII, all anti-FN sera were absorbed with purified plasma factor VIII before use. These absorptions and the patterns of mobility of the [^{3}H] FN peaks seen in Fig. 4 rule out the possibility that the isolated radioactive peaks seen in Figs. 4B and 4C are factor VIII antigen.

FN is one of the major proteins synthesized and secreted by cultured endothelial cells. It appears to constitute ~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 fraction (separated by Sepharose 6B) prepared from the same starting material (31) and thus probably represented <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, i.e. factor VIII antigen ~5–10 μg/ml and plasma FN ~330 μg/ml (1). Cultures of human endothelial cells secreted (Table I) an average of 51.8 μg FN/25 cm^2 dish during 3 days in culture. By comparison, a variety of first passage human embryonic cell strains secreted an average of 106 μg FN/25 cm^2 dish (equivalent) during 3 days in culture (15, 41). Thus, endothelial cells synthesize and release into their culture medium significant amounts of FN having subunits of the same molecular weight as those found in human plasma.

FN has been shown to be present in the endothelium and subendothelium of a variety of blood vessels. Linder et al. (7) in studies on the developing chick embryo showed that in addition to primitive mesenchymal tissue and basal lamina, endothelial cells and the subendotheium of arteries and veins and also of brain capillaries contained FN when tissue sections were studied by immunofluorescence microscopy. FN was absent in parenchymal cells in a variety of organs such as the liver, striated and smooth muscle, brain (except neural sheaths), cartilage, and bone. Similar immunofluorescence studies on a variety

of human tissues have shown that FN is most prominently seen associated with endothelial cells (28, 29). In addition, FN was also seen in basal lamina, the media of blood vessels, and loose connective tissue (29). Our present data in conjunction with these immunofluorescence studies and the location of endothelial cells (adjacent to the blood stream) suggest that endothelial cells are a major site of synthesis of plasma FN.

A physiologic role of FN in plasma has just been demonstrated. FN has shown to be identical to human α_2 opsonic glycoprotein which is thought to have the important restorative function of mediating the uptake of colloids and intravascular particulates by the reticuloendothelial system (1, 2, 42, 43). Our in vitro study and the immunofluorescence studies performed on tissue sections by others (7, 28, 29) have shown that the extracellular matrix of endothelial cells contains FN. The physiologic role of FN in tissues has been inferred from its effects on cultured cells. FN is necessary for the attachment of tissue culture cells to culture vessels (19–22). FN also increases the strength of attachment of transformed cells to culture vessels and thus changes their morphology (though not their growth characteristics and malignant potential) (24, 25). The mechanism of these two effects is unknown. Fibroblast cultures contain fibrils which stain for both FN and collagen when examined by immunofluorescence microscopy (17). The basis for this congruence of location may be the recently described noncovalent interactions of FN with other FN molecules (44) and with collagen (18). FN crosslinks with itself and other proteins through disulfide links (9, 45). FN on cell surfaces is known to be immobile and may serve as an anchorage point for cell attachment (44). Thus, FN in the subendothelium may be an important structural component which plays a role in endothelial cell attachment and adhesiveness under normal conditions and after injury to the vascular wall.

Summary

Plasma fibronectin is probably the major nonimmune particulate opsonin in blood and is cross-linked to fibrin during the final stage of blood coagulation. Fibronectin also occurs in an insoluble form in basement membranes especially those underlying endothelial cells and in loose connective tissue.

Fibronectin was demonstrated in cultured human endothelial cells and in the surrounding extracellular matrix by immunofluorescence microscopy by using antibody to human plasma fibronectin. Cultured human endothelial cells released fibronectin into the culture medium which was immunologically identical to the fibronectin in human plasma. Cultured human endothelial cells were labeled with [^{3}H] leucine. The radioactive fibronectin present in the endothelial postculture medium and in urea extracts of cellular monolayers was isolated with either anti-fibronectin coupled to Protein A-Sepharose or double antibody immunoprecipitation and characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. When reduced, the [^{3}H] fibronectin synthesized by cultured endothelial cells had the same mol wt (~200,000) as plasma fibronectin. Unreduced, the [^{3}H] fibronectin synthesized by endothelial cells migrated as a dimer, as did plasma fibronectin. Fibronectin accounted for ~15% of the protein synthesized and released by endothelial cells into the culture medium.

Thus, cultured endothelial cells synthesize fibronectin, secrete it into the culture medium, and incorporate it into extracellular matrix. The results suggest that the endothelial cell is potentially a major site of synthesis of circulating plasma fibronectin. In addition, fibronectin derived from endothelial cells may be an important structural component of the subendothelium.

The authors wish to thank Lilane Bronson, Christine Baranowski, and Louise Wolf for their excellent technical assistance.

Received for publication 23 February 1978.

References

1. Blumenstock, F., P. Weber, and T. M. Saba. 1977. Isolation and biochemical characterization of α-2-opsonic glycoprotein from rat serum. *J. Biol. Chem.* **252**:7156.
2. Blumenstock, F. A., T. M. Saba, P. Weber, and R. Laffin. 1978. Biochemical and immunological characterization of human opsonin α_2 SB glycoprotein; its identity with cold insoluble globulin. *J. Biol. Chem.* In press.
3. Mosher, D. F. 1975. Cross-linking of cold-insoluble globulin by fibrin-stabilizing factor. *J. Biol. Chem.* **250**:6614.
4. Mosher, D. F. 1976. Action of fibrin-stabilizing factor on cold-insoluble globulin and α_2-macroglobulin in clotting plasma. *J. Biol. Chem.* **251**:1639.
5. Morrison, P. R., J. T. Edsall, and S. G. Miller. 1948. Preparation and properties of serum and plasma proteins. XVIII. The separation of purified fibrinogen from fraction 1 of human plasma. *J. Am. Chem. Soc.* **70**:3103.
6. Mosesson, M. W., and R. A. Umfleet. 1970. The cold-insoluble globulin of human plasma. I. Purification, primary characterization, and relationship to fibrinogen and other cold-insoluble fraction components. *J. Biol. Chem.* **245**:5728.
7. Linder, E., A. Vaheri, E. Ruoslahti, and J. Wartiovaara. 1975. Distribution of fibroblast surface antigen in the developing chick embryo. *J. Exp. Med.* **142**:41.
8. Ruoslahti, E., and A. Vaheri. 1975. Interaction of soluble fibroblast surface antigen with fibrinogen and fibrin. Identity with cold insoluble globulin of human plasma. *J. Exp. Med.* **141**:497.
9. Keski-Oja, J., D. F. Mosher, and A. Vaheri. 1977. Dimeric character of fibronectin, a major cell surface associated glycoprotein. *Biochem. Biophys. Res. Commun.* **74**:699.
10. Hynes, R. O., and J. M. Bye. 1974. Density and cell cycle dependence of cell surface proteins in hamster fibroblasts. *Cell.* **3**:113.
11. Yamada, K. M., and J. A. Weston. 1974. Isolation of a major cell surface glycoprotein from fibroblasts. *Proc. Natl. Acad. Sci. U.S.A.* **71**:3492.
12. Ruoslahti, E., A. Vaheri, P. Kuusela, and E. Linder. 1973. Fibroblast surface antigen: a new serum protein. *Biochim. Biophys. Acta.* **322**:352.
13. Kuusela, P., E. Ruoslahti, and A. Vaheri. 1975. Polypeptides of a glycoprotein antigen (SF) present in serum and surface of normal but not of transformed chick fibroblasts. *Biochim. Biophys. Acta.* **379**:295.
14. Vaheri, A., and E. Ruoslahti. 1975. Fibroblast surface antigen produced but not retained by virus-transformed human cells. *J. Exp. Med.* **142**:530.
15. Mosher, D. F., O. Saksela, J. Keski-Oja, and A. Vaheri. 1977. Distribution of a major surface-associated glycoprotein, fibronectin, in cultures of adherent cells. *J. Supramol. Struct.* **6**:551.
16. Vaheri, A., E. Ruoslahti, B. Westermark, and J. Pontén. 1976. A common cell-type specific surface antigen in cultured human glial cells and fibroblasts: loss in malignant cells. *J. Exp. Med.* **143**:64.

17. Bornstein, P., and J. F. Ash. 1977. Cell surface-associated structural proteins in connective tissue cells. *Proc. Natl. Acad. Sci. U.S.A.* **74**:2480.
18. Engvall, E., and E. Ruoslahti. 1977. Binding of soluble form of fibroblast surface protein, fibronectin, to collagen. *Int. J. Cancer.* **20**:1.
19. Klebe, R. J. 1974. Isolation of a collagen-dependent cell attachment factor. *Nature (Lond.).* **250**:248.
20. Jilek, F., and H. Hörmann. 1977. Cold insoluble globulin. II. Plasminolysis of cold insoluble globulin. *Hoppe-Seyler's Z. Physiol. Chem.* **358**:133.
21. Hook, M., K. Rubin, A. Oldberg, B. Öbrink, and A. Vaheri. 1977. Cold insoluble globulin mediates the adhesion of rat liver cells to plastic petri dishes. *Biochem. Biophys. Res. Commun.* **79**:726.
22. Pearlstein, E. 1976. Plasma membrane glycoprotein which mediates adhesion of fibroblasts to collagen. *Nature (Lond.).* **262**:497.
23. Hynes, R. O. 1976. Cell surface proteins and malignant transformation. *Biochim. Biophys. Acta.* **458**:73.
24. Yamada, K. M., S. S. Yamada, and I. Pastan. 1976. Cell surface protein partially restores morphology, adhesiveness, and contact inhibition of movement to transformed fibroblasts. *Proc. Natl. Acad. Sci. U.S.A.* **73**:1217.
25. Ali, I. U., V. Mautner, R. Lanza, and R. O. Hynes. 1977. Restoration of normal morphology, adhesion, and cytoskeleton in transformed cells by addition of a transformation-sensitive surface protein. *Cell.* **11**:115.
26. Jaffe, E. A., C. R. Minick, B. Adelman, C. G. Becker, and R. Nachman. 1976. Synthesis of basement membrane collagen by cultured human endothelial cells. *J. Exp. Med.* **144**:209.
27. Howard, B. V., E. J. Macarak, D. Gunson, and N. A. Kefalides. 1976. Characterization of the collagen synthesized by endothelial cells in culture. *Proc. Natl. Acad. Sci. U.S.A.* **73**:2361.
28. Vaheri, A., D. F. Mosher, J. Wartiovaara, J. Keski-Oja, M. Kurkinen, and S. Stenman. 1977. Interactions of fibronectin, a cell-type specific surface-associated glycoprotein, *In* Cell Interactions in Differentiation. M. Karkinen-Jääskeläinen, L. Saxén, and L. Weiss, editors. Academic Press, Inc., New York. p. 311.
29. Stenman, S., and A. Vaheri. 1978. Distribution of a major connective tissue protein, fibronectin, in normal human tissues. *J. Exp. Med.* **147**:1054.
30. Jaffe, E. A., R. L. Nachman, C. G. Becker, and C. R. Minick. 1973. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J. Clin. Invest.* **52**:2745.
31. Jaffe, E. A., L. W. Hoyer, and R. L. Nachman. 1973. Synthesis of antihemophilic factor antigen by cultured human endothelial cells. *J. Clin. Invest.* **52**:2757.
32. Becker, C. G., and P. C. Harpel. 1976. α_2-Macroglobulin on human vascular endothelium. *J. Exp. Med.* **144**:1.
33. Jaffe, E. A., L. W. Hoyer, and R. L. Nachman. 1974. Synthesis of von Willebrand factor by cultured human endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.* **71**:1906.
34. Weksler, B. B., T. Chang, and C. Ley. 1977. Convenient microtiter assay for von Willebrand's factor. *Thromb. Haemostasis.* **38**:350.
35. Jaffe, E. A., and R. L. Nachman. 1975. Subunit structure of factor VIII antigen synthesized by cultured human endothelial cells. *J. Clin. Invest.* **56**:698.
36. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265.
37. Mancini, G., A. O. Carbonara, and J. F. Heremans. 1965. Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochemistry.* **2**:234.
38. Yamada, K. M., S. S. Yamada, and I. Pastan. 1975. The major cell surface

glycoprotein of chick embryo fibroblasts is an agglutinin. *Proc. Natl. Acad. Sci. U.S.A.* **72**:3158.

39. Kessler, S. W. 1975. Rapid isolation of antigens from cells with a staphylococcal Protein A-antibody absorbent: parameters of the interaction of antibody-antigen complexes with Protein A. *J. Immunol.* **15**:1617.
40. Weinstein, M. J., D. Deykin, and E. W. Davie. 1976. Quantitative determination of factor VIII protein by two-stage gel electrophoresis. *Br. J. Haematol.* **33**:343.
41. Mosher, D. F., O. Saksela, and A. Vaheri. 1977. Synthesis and secretion of alpha-2-macroglobulin by cultured adherent lung cells. Comparison with cell strains derived from other tissues. *J. Clin. Invest.* **60**:1036.
42. Blumenstock, F., P. Weber, T. M. Saba, and R. Laffin. 1977. Electroimmunoassay of alpha-2-opsonic protein during reticuloendothelial blockade. *Am. J. Physiol.* **232**:R80.
43. Saba, T. M. 1975. Reticuloendothelial systemic host defense after surgery and traumatic shock. *Circ. Shock.* **2**:91.
44. Schlesinger, J., L. S. Barak, G. G. Hammes, K. M. Yamada, I. Pastan, W. W. Webb, and E. L. Elson. 1977. Mobility and distribution of a cell surface glycoprotein and its interactions with other membrane components. *Proc. Natl. Acad. Sci. U.S.A.* **74**:2909.
45. Hynes, R. O., and A. Destree. 1977. Extensive disulfide bonding at the mammalian cell surface. *Proc. Natl. Acad. Sci. U.S.A.* **74**:2855.