Human Endothelial Cells Synthesize, Process, and Secrete Fibronectin Molecules Bearing an Alternatively Spliced Type III Homology (ED1)

By John H. Peters, Lee Ann Sporn, Mark H. Ginsberg, and Denisa D. Wagner

Collagen fibronectin (Fn) bearing an alternatively spliced extra type III structural repeat (ED1) is normally present at low concentrations in blood plasma. The source of this material remains uncertain. In this study, primary cultures of human umbilical vein endothelial cells (HUVEC) labeled with $^{38}$S-methionine were observed to synthesize Fn monomers both with and without this segment. Monomers isolated from cell lysates with antibodies to the ED1 sequence comigrated in nonreduced sodium dodecyl sulfate polyacrylamide gel electrophoresis with the slower (designated M1), but not the faster (designated M2), of two major monomeric populations that were recognized by antibodies raised to plasma-derived Fn. The differences between M1 and M2 were not due to glycosylation, since they were also observed between species of Fn monomer purified from cells grown in the presence of tunicamycin. M1 and M2 were both observed to incorporate with a similar rate into dimeric Fn, indicating that Fn monomers with and without the ED1 site can dimerize with similar efficiency. Analysis of reduced samples of Fn isolated from cells with anti-ED1 antibodies indicated the presence of both M1-M1 and M1-M2 dimers. In addition to being incorporated into extracellular matrix, ED1+ Fn was observed to be secreted in soluble form into the medium, potentially reflecting intravascular release of this protein by endothelial cells in vivo.

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MATERIALS AND METHODS

Cells and cell culture conditions. Primary cultures of endothelial cells were obtained from human umbilical veins by mild proteolytic digestion as described, and cultured in McCoy’s 5A medium (Flow Laboratories, McLean, VA) containing 20% fetal bovine serum. For continuous labeling, cells were grown in the presence of L-$^{38}$S-methionine (New England Nuclear, Boston, MA) at 20 μCi/mL. For pulse labeling, confluent cells were washed twice with Hank’s balanced salt solution and incubated for 15 minutes at 37°C with media lacking serum. The cells were then processed for metabolic labeling studies.

From the Department of Immunology, Research Institute of Scripps Clinic, La Jolla; Pulmonary Division, Department of Medicine, University of California, San Diego; Hematology Unit, Department of Medicine, University of Rochester School of Medicine and Dentistry, NY; and Hematology Division, Department of Medicine, Tufts University School of Medicine, Boston, MA

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Address reprint requests to John H. Peters, MD, Center for Cancer Research, E17-225, Massachusetts Institute of Technology, 77 Massachusetts Ave, Cambridge, MA 02139.

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covered with L-(15)Smethionine at 0.8 to 1.5 mCi/mL in media without serum for periods varying from 5 to 20 minutes. After pulse labeling, the cells were washed once with complete media and then again in Hank's solution, and lysed immediately or covered with fresh unlabelled complete media for varying chase periods at 37°C.

**Antiserum.** The preparation and characterization of goat antiserum raised against a 29-amino acid synthetic peptide of the ED1 region of human Fn and against Fn derived from rabbit plasma were described previously. The goat antiserum to rabbit plasma Fn was crossreactive with human Fn as judged by Western blotting and enzyme-linked immunosorbent assays (ELISA).

**Monoclonal antibody S2/54.** Balb C mice were immunized with the 29-amino acid synthetic ED1 peptide-KLH conjugate used to raise antiserum to the ED1 in a goat. The priming dose of antigen was 25 μg emulsified in complete Freund's adjuvant and injected subcutaneously. This was followed in 18, 25, and 32 days by intraperitoneal injections of 25-μg antigen. Sera were then checked for preferential antibody titers for cellular versus plasma-derived Fns in ELISA. Parallel microtiter wells were coated either with Fn (3 μg/mL in 0.1 mol/L NaHCO₃) purified by gelatin chromatography from the media of GM-1380 fibroblasts grown in the presence of Fn-depleted fetal bovine serum or from human plasma. Duplicate dilutions of sera were then applied to the Fn-coated wells, followed after a washing step with peroxidase-conjugated goat antibodies to mouse immunoglobulins (Igs) (Tago Inc, Burlingame, CA). One mouse had serum which, diluted 1:100,000, bound to wells coated with cellular but not plasma Fn. On the 46th day after initial immunization, it was given an intravenous booster of 50-μg antigen. Five days later the spleen was taken and the washed spleen cell suspension was fused with SP-2 myeloma cells at a 5:1 ratio. The fused cells were grown in hypoxanthine-aminopterin-thymidine medium before distribution in microtiter wells. Fifty-one colonies of the resulting hybridomas expressing antibody specific for cellular Fn were grown in the presence of hypoxanthine and aminopterin before being screened by ELISA. Parallel microtiter wells were coated either with Fn, Fn-depleted fetal bovine serum, or plasma Fn. After incubation with samples of cell lysate or media for 90 minutes at room temperature, each sample of cell lysate was divided such that the final concentration of ingredients was that of the buffer used for labeling. The cells were washed once with complete media and then again in Hank's solution, and lysed immediately or covered with fresh unlabelled complete media for varying chase periods at 37°C.

**ScreenType ELISA kit (Boehringer Mannheim Biochemicals, Indianapolis, IN), supernatant from this clone contained both IgGl and IgG2b antibodies, both with k light chains. Despite a third subcloning step at limiting dilutions of 0.5 cell per well, in no cases were the antibodies to rabbit IgG to stain the total pool of extracellular Fn.**

**Results**

**Intracellular processing of Fn.** Newly formed Fn monomers have been shown to incorporate into dimers within minutes of synthesis in fibroblasts. Therefore, we initiated our analysis of the synthesis and processing of Fn polypeptides in primary cultures of HUVEC by pulse-labeling these cells with 15S-methionine for a 10-minute period immediately followed by lysis. The total pool of Fn immunopurified from a portion of the resulting lysate with antibodies to plasma-derived Fn was comprised of three distinct bands when the volume was incubated with beads (20 μg/mL media) that had been preincubated with 5.9 μL/mg antiserum to the ED1 segment, while the remaining 20% was incubated with beads (80 μg/mL media) that had been preincubated with 1.3 μL/mg antiserum to plasma-derived Fn. After incubation, the beads were washed extensively and boiled in electrophoresis sample buffer to elute bound Fns for analysis by gel electrophoresis. These procedures did not result in quantitative removal of either ED1 + Fn or total Fn from samples, as judged by the capacity of a second application of each method to yield additional labeled material possessing identical electrophoretic mobility to that obtained with either antibody in the first instance. To demonstrate that immunopurification of labelled ED1 + Fn from culture medium was specific, Protein A beads were split in half after preincubation with anti-ED1 antiserum. One resulting portion was preincubated for 1 hour at room temperature with 1.2 mL of synthetic ED1 peptide (167 μg/mL) in phosphate-buffered saline (PBS) corrected to a pH of 7.4 with NaOH, while the other was incubated with the same volume of PBS (pH 7.4) alone. Equal volumes of media obtained from cells that had been subjected to continuous labeling for 3 days were then added to each of the two portions of beads (plus or minus peptide) and incubated as described above.

**Electrophoresis gels.** Five percent sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels were prepared as described by Laemmli. The 29-amino acid synthetic peptide used to raise antiserum to the ED1 in a goat was purified for gel electrophoresis. This was followed by autoradiography, and the radiolabeled species of Fn within samples that had been immunopurified with either anti-ED1 or anti-plasma Fn antibodies were quantified by densitometry.

**Tunicamycin treatment.** To assess the effect of inhibition of N-linked glycosylation on newly synthesized Fn, confluent layers of HUVEC were grown in the presence of culture medium containing a final concentration of 1 μg/mL tunicamycin (Calbiochem, San Diego, CA), using a fresh made stock solution at 1 mg/mL in DMSO. The cells were incubated with tunicamycin for 15 hours before pulse labeling experiments, as well as during the period of labeling.

**Endoglycosidase H digestion.** Purified Fn was diluted in 0.1 mol/L Tris buffer, pH 5.8, so that the final concentration of SDS was less than 0.5%. Endoglycosidase H (Calbiochem) was added (3 μg/mL) and samples were incubated for 2 hours at 37°C before analysis on gels.

**Immunofluorescence.** Cells to be examined were cultured entirely in media containing fetal bovine serum that had been depleted of Fn by passage of 500 mL serum over a 100-mL column of gelatin sepharose. Cells fixation, permeabilization, and fluorescent staining were performed as previously described. Individual coverslips were double-stained with (1) McAb S2/54 ascites (1:10 dilution) followed by rhodamine-conjugated goat antibodies to mouse IgG to detect ED1 + Fn; and (2) rabbit anti-human plasma Fn antiserum (1:100 dilution) (Calbiochem) followed by fluorescein-conjugated goat antibodies to rabbit IgG to stain the total pool of extracellular Fn.
analyzed nonreduced in SDS-PAGE (Fig 1, lane 1). All three bands migrated more slowly than the 200-Kd myosin molecular weight standard. The slowest migrating of these comigrated with Fn immunopurified from the medium of HUVEC that had been subjected to 3 days of continuous labeling (not shown), indicating that this band represented dimeric Fn. The two faster-migrating bands, designated M1 and M2, corresponded to monomeric Fn. Fn immunopurified from the companion fraction of cell lysate with antibodies to the ED1 segment contained only material comigrating with dimeric Fn and the slower-migrating of the two monomeric species (Fig 1, lane 2). When subjected to gel analysis reduced, the three species comprising total Fn resolved into two major bands that migrated more slowly than the two monomeric species observed under nonreducing conditions (Fig 1, lane 3), indicating that at least some intrachain disulfide bonds formed before dimerization of Fn monomers. Although antibodies to the ED1 segment isolated Fn that comigrated primarily with the slower migrating population in reduced electrophoresis (Fig 1, lane 4), densitometric analysis uniformly showed that a portion (approximately 10%) of this material comigrated with the faster migrating of the two species (see Fig 4).

It was possible that the difference in apparent molecular mass between M1 and M2 might result from differential carbohydrate processing, since Fn is a glycoprotein containing 5% to 7% carbohydrate, most of which appears to be in asparagine-linked side chains. To address this possibility, cells were pulse-labeled after having been cultured in the presence of tunicamycin, which inhibits the addition of asparagine-linked carbohydrate side chains. The two major monomeric species of Fn, which were immunopurified from cells cultured in the presence of this agent for 15 hours before and during a 20-minute exposure to 3S-methionine, showed similar increments in rate of electrophoretic migration nonreduced (Fig 2, left panel), and reduced (Fig 2, right panel), indicating that these species do not differ in size or antigenicity based on content of N-linked carbohydrate.

To further examine the relationship between the two apparent monomeric species of Fn (M1 and M2) in HUVEC, pulse-chase studies were conducted. Cells were exposed to 3S-methionine for 5 minutes, then immediately lysed or incubated in unlabeled medium for 5 or 25 minutes before lysis. With increasing periods of chase, greater quantities of label were incorporated into dimeric Fn, whereas lesser quantities were present in M1 and M2, until the latter species were no longer evident by 25 minutes of chase (Fig 3A). When the monomeric bands, which were observed under nonreducing conditions at 0-, 5-, and 25-minute chase timepoints, were quantified by densitometry and plotted versus time, the radioactivity incorporated into M1 and M2 was observed to diminish at similar rates (not shown). Of note, no labeled Fn could be immunopurified from the medium of cells at the 25-minute chase timepoint, indicating that the newly synthesized Fn dimers were still associated with the cells.

The entirely dimeric intracellular pool of labeled Fn that was seen at late chase timepoints (Fig 3A, 25-minute chase) included slightly slower migrating material not present at early chase timepoints (Fig 3A, 5-minute chase). The appearance of this material coincided with loss of resolution of two major Fn subunit populations in reduced gel analysis (Fig 3B, 25-minute chase). In contrast to the faster migrating dimers evident at this and earlier chase times, the newly appearing dimeric material was resistant to endoglycosidase H, which cleaves the chitobiosyl unit of high mannose but not complex carbohydrate side chains (not shown), indicating that the appearance of this material at this time resulted from processing of carbohydrate side chains from high mannose to complex form.

**The composition of intracellular Fn dimers.** As can be seen by comparison of Figs 1 and 2, in which Fn was...
immunopurified from cells labeled for 10 or 20 minutes, respectively, the labeled intracellular pool of Fn was observed to undergo a major shift between timepoints toward dimeric as opposed to monomeric forms. This shift was confirmed by densitometric analysis (Fig 4, left panels). Although the labeled Fn that was isolated by anti-ED1 antibodies at either 10 or 20 minutes did not contain free M2 monomers, this material dissociated on reduction to yield a small portion of M2 subunits at both timepoints (Fig 4, lower right panel), indicating the presence within cells of M1-M2 heterodimers. Of note, the primarily dimeric material isolated with anti-ED1 antibodies at the 20-minute timepoint resolved on reduction into relatively greater proportions of M1 and lesser proportions of M2 subunits than the corresponding material isolated with antibodies to plasma-derived Fn (Fig 4, upper right panel).

Secretion of ED1 + Fn. For endothelial cells to be a source of soluble ED1 + Fn circulating in the blood plasma,
they should have the capacity both to synthesize and secrete this material in soluble forms. Therefore, we examined the capacity of cultured endothelial cells to secrete soluble ED1+ Fn after synthesis. Medium was collected from primary cultures of HUVEC after 3 days of continuous labeling with 35S-methionine, and portions were subjected to immunopurification either with antiserum to plasma-derived Fn or to the ED1 segment. As shown in Fig 5, material of similar electrophoretic mobility, constituting a diffuse band in reduced electrophoresis, was purified by both types of antibody. Immunopurification of labeled Fn by antibodies to the ED1 segment was blocked by coincubation of the Protein A beads with synthetic ED1 peptide before and during immunopurification, indicating that purification of ED1+ Fn was specific. Therefore, cultured HUVEC secrete soluble ED1+ Fn into the medium after synthesis. Of note, the total pool of labeled Fn that was secreted into the medium by these cells migrated slightly slower in nonreduced and reduced electrophoresis than Fn isolated by gelatin affinity chromatography from human plasma (not shown).

Matrix incorporation of ED1+ Fn. To examine the capacity of endothelial cells to incorporate endogenous ED1+ Fn into their extracellular matrix, monoclonal anti-ED1 peptide antibodies were used to examine the subcellular matrix of primary cultures of HUVEC grown in media containing fetal bovine serum depleted of Fn. As shown in Fig 6, ED1+ Fn was specifically detected by immunofluorescence microscopy in a fibrillar pattern within the extracellular matrix of HUVEC, and this pattern was substantially similar to that detected with antibodies raised to plasma-derived Fn.

Discussion

Forms of Fn bearing the ED1 segment appear by immunohistochemical analysis to be confined mainly to the endothelium of larger blood vessels in normal adult tissues. This observation, coupled with the close association between endothelial cells and the bloodstream, has led to speculation that the small quantities of ED1+ Fn that are normally present in plasma may be produced by these cells.

Our observations strengthen this hypothesis, since we have found that primary cultures of HUVEC synthesize, secrete, and deposit ED1+ Fn within their extracellular matrix. However, these cells do not produce only Fn bearing the ED1 site. We have observed that HUVEC synthesize two major populations of Fn monomer (designated M1 and M2), which differ in electrophoretic mobility. Only the M1 species was observed to contain molecules bearing the ED1 site, indicating that the greater apparent molecular mass of this population can be attributed at least in part to inclusion of molecules bearing this alternatively spliced site. The observation that HUVEC synthesize monomers both with and without the ED1 site is consistent with previous studies in which other types of cells have been shown to contain species of Fn mRNA differing in content of the sequence encoding this site, and with the finding that fibroblasts synthesize Fn subunits both with and without the ED1 segment.
Both populations of Fn monomer were observed to possess rates of electrophoretic migration that were faster under nonreducing as compared with reducing conditions, consistent with early acquisition by Fn polypeptides of intrachain disulfide bonds in the endoplasmic reticulum preceding dimerization. Of interest, the three regions of Fn that are subject to alternative splicing at the level of the primary transcript with the potential to generate as many as 20 different monomeric forms, \(1^4,5\) the observation that primary cultures of HUVEC synthesize Fn monomers that resolve into two major species in one-dimensional electrophoresis was unexpected. However, there is precedent for this finding. Hepatocyte-derived Fn, the predominant form in plasma, classically resolves in reduced electrophoresis into a doublet comprised of two distinct bands corresponding with two populations of mature subunits. Analogous to the situation for M2, the smaller of the two major Fn subunit populations present in the plasma of rodents can be distinguished from its larger counterpart by an absence of molecules bearing a specific alternatively spliced sequence, in this case localized to the IIIICS region. \(1^6\) Although the two species of monomer that we have observed to be present in HUVEC may each be heterogeneous, potentially including differently spliced subspecies of Fn not resolved by the gel system used, it is of interest to note that we have used this same system to identify as many as four species of Fn monomer, at least two of which (within 10 minutes after initiation of synthesis). However, differences in size and antigenicity between the two species were not based on their content of such carbohydrates, since these relative differences remained between species immunopurified from cells grown in the presence of tunicamycin, which inhibits the biosynthesis of the dolichol-linked precursor of N-linked side chains. \(23^\) After addition of carbohydrate, the two populations of monomer were both observed to incorporate simultaneously and completely into Fn dimers of similar electrophoretic mobility. The latter observation indicates that: (1) the M2 population is not a degradation product of the M1 population; and (2) monomers with and without the ED1 segment can assemble into dimers with similar efficiency.

The observation that the labeled material isolated from cells with anti-ED1 antibodies resolved into only dimers and M1 monomers in nonreduced electrophoresis, yet on reduction included some M2 subunits, indicates that some of the dimers isolated by these antibodies were composed of an M1 subunit linked to an M2 subunit. Therefore, M1-M2 dimers bearing only one ED1 site are assembled within HUVEC. Still, the relative scarcity of M2 subunits within the reduced material immunopurified with anti-ED1 antibodies at the 20-minute timepoint (Fig 4, lower right panel) indicated that M1-M2 dimers comprised only a small portion of the primarily dimeric material isolated. Therefore, the balance of dimers within this material must have been of the M1-M1 variety. The prevalence of M1 subunits within dimers immunopurified with anti-ED1 antibodies can probably be attributed to the apparently greater quantities of M1 as compared with M2 monomers available for dimerization within cells (Fig 4, upper panels), and the capacity of antibodies to the ED1 segment to recognize only dimers of M1-M1 and M1-M2 (but not of M2-M2) composition. Of note, M1-M1 dimers isolated from cells with anti-ED1 antibodies may have contained either one or two ED1 sites since, in the absence of quantitative immunopurification, only M1 monomers purified with these antibodies can be claimed a priori to uniformly bear the ED1 segment.

Because the human Fn gene contains at least three sites that are subject to alternative splicing at the level of the primary transcript with the potential to generate as many as 20 different monomeric forms, \(1^4,5\) the observation that primary cultures of HUVEC synthesize Fn monomers that resolve into two major species in one-dimensional electrophoresis was unexpected. However, there is precedent for this finding. Hepatocyte-derived Fn, the predominant form in plasma, classically resolves in reduced electrophoresis into a doublet comprised of two distinct bands corresponding with two populations of mature subunits. Analogous to the situation for M2, the smaller of the two major Fn subunit populations present in the plasma of rodents can be distinguished from its larger counterpart by an absence of molecules bearing a specific alternatively spliced sequence, in this case localized to the IIIICS region. \(1^6\) Although the two species of monomer that we have observed to be present in HUVEC may each be heterogeneous, potentially including differently spliced subspecies of Fn not resolved by the gel system used, it is of interest to note that we have used this same system to identify as many as four species of Fn monomer, at least two of which...
of which contained molecules bearing the ED1 segment, in similar pulse-chase experiments with human fetal foreskin fibroblasts (our unpublished results). Therefore, the population of Fn monomers synthesized by primary cultures of HUVEC appears to be simpler than that which has been demonstrated to be produced by fibroblasts.

In this qualitative study, we have found that primary cultures of human umbilical vein endothelial cells synthesize, secrete, and deposit forms of Fn bearing the ED1 segment. The extent to which vascular endothelial cells might similarly produce this form of Fn in vivo, thereby potentially contributing to the circulating pool of ED1 + Fn, will require further experimentation. Although the significant of inclusion of ED segments in circulating Fn remains uncertain, these sites could potentially confer specific functions, particularly in view of recent observations indicating that the alternatively spliced type III connecting segment of Fn contains cell type-specific adhesion sites.

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