Transglutaminase-Mediated Processing of Fibronectin by Endothelial Cell Monolayers†

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ABSTRACT: We studied the interaction of [^{125}I]fibronectin with human umbilical vein endothelial cells. Endothelial cell monolayers cross-linked [^{125}I]fibronectin which had been preadsorbed to gelatin-coated dishes. The cross-linking of the substrate-immobilized [^{125}I]fibronectin was mediated by cell-associated tissue transglutaminase and occurred more rapidly during the first 30 min after endothelial cell seeding but also continued for several hours after the cells were fully spread. The processing of the [^{125}I]fibronectin was associated with the basolateral surface of the endothelial cell, as demonstrated by the finding that cross-linking did not occur when [^{125}I]fibronectin was presented to the apical surface of confluent monolayers. Transglutaminase activity was not necessary for attachment and spreading of HUVEC on a fibronectin/gelatin matrix. The presence of a nonpeptidyl transglutaminase inactivator rendered the cells more susceptible to detachment by trypsin and destabilized the association of fibronectin with the subendothelial extracellular matrix. Thus, endothelial cells process fibronectin into cross-linked multimers due to the expression of tissue transglutaminase at the basal surface of the cell. This process may serve to stabilize the extracellular matrix and to firmly anchor the cells to the basement membrane.

The extracellular matrix of vascular endothelium plays an essential role in mediating biochemical events in endothelial cells which influence a variety of biological processes (Hynes, 1990; Ruoslahti, 1988; Albelda et al., 1989; Ingber, 1990). The attachment and spreading of human endothelial cells and fibroblasts on tissue culture dishes coated with matrix components such as fibronectin, laminin, type-IV collagen, vitronectin, or von Willebrand factor are largely mediated by the interaction of cellular receptors, known as integrins, with specific functional domains of each matrix protein. In the case of fibronectin, this association is principally accomplished by the interaction of its cell-binding domain with the $\alpha_5\beta_1$ integrin, an interaction which is involved in regulating organization of the cytoskeleton, in determining cell morphology, and in controlling cell growth (Hynes, 1990; Ruoslahti, 1988; Albelda et al., 1989; Ingber, 1990).

The fibronectin molecule is a dimeric glycoprotein, each subunit having a molecular mass of 230–250 kDa and expressing heterogeneity due to differential splicing at three distinct sites (Hynes, 1990; Ruoslahti, 1988). The C-terminal half of the molecule contains the cell-binding domain, within which is located the Arg-Gly-Asp peptide sequence, which mediates the interaction of fibronectin with the $\alpha_5\beta_1$ integrin expressed on the surface of many different types of cells (Hynes, 1990; Ruoslahti, 1988; Albelda et al., 1989; Ingber, 1990; Yamada, 1991). Other sequences located at the C-terminus also participate in the binding of fibronectin to other integrins as well as to proteoglycans (Yamada, 1991; LeBaron et al., 1988; McCarthy et al., 1990). At the N-terminus, the fibronectin molecule contains domains which mediate its binding to collagen or gelatin and also to fibrin, heparin, and a fibroblast matrix assembly receptor (Ruoslahti, 1988, 1989; McDonald, 1988; Mosher et al., 1991). The N-terminal region of fibronectin also contains distinct binding sites and substrate cross-linking sites both for the plasma transglutaminase known as factor XIIIa (McDonagh et al., 1981) and for tissue transglutaminase, which is present in a wide variety of cell types (LeMosy et al., 1992; Fesus et al., 1986).

Tissue transglutaminase is a calcium-dependent enzyme which mediates covalent cross-linking reactions between a limited number of proteins by forming amide bonds between the γ-carboxamide groups of specific peptide-bound glutamine residues and the ϵ-amino groups of particular peptide-bound lysines (Folk, 1980; Lorand & Conrad, 1984; Ichinose et al., 1990; Greenberg et al., 1991). Although it is thought that the enzyme serves predominantly an intracellular function, tissue transglutaminase can be expressed on the extracellular surface of cells (Barsigian et al., 1988; Martinez et al., 1989; Barsigian et al., 1991), and several extracellular matrix components, including fibronectin, fibrin, collagen, vitronectin, and von Willebrand factor, are known transglutaminase substrates (Greenberg et al., 1991). Recent evidence suggests that it may play a role in stabilizing the interactions between cells and the extracellular matrix (Ikura et al., 1988; Gentile et al., 1992), since cells deficient in tissue transglutaminase are less firmly attached to matrix proteins than are the same cells following transfection with the tissue transglutaminase gene (Gentile et al., 1992).

In the present communication, we report that human endothelial cells of either venous or arterial origin process substrate-immobilized fibronectin into high molecular weight covalently cross-linked multimers via a reaction which is mediated by tissue transglutaminase functionally expressed at the basal surface of the endothelial cell monolayer. This process is operative during the initial attachment and spreading stage as well as in a continuous fashion following the formation

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of a confluent monolayer. A nonpeptidyl transglutaminase inactivator, which inhibited the extracellular cross-linking of matrix-associated fibronectin by endothelial cells, increased the sensitivity of the cells to trypsin-induced detachment and destabilized the association of fibronectin itself with the subendothelial matrix. Thus, transglutaminase may play a role in reinforcing endothelial cell–matrix interactions, and this may be due in part to the cross-linking of matrix fibronectin.

EXPERIMENTAL PROCEDURES

Materials. Carrier-free ^{125}I (NaI in NaOH) was obtained from Ventrex Division of Hycor Biomedical, Inc. (Portland, MA). Triton X-100 was from Amersham Corp. Corning plastic 12-well (22-mm diameter) tissue culture-treated polystyrene dishes were purchased from Fisher. Acrylamide and bisacrylamide were from Bio-Rad. Media 199 with Earle's salts (catalog no. 380–2340AJ), penicillin/streptomycin, L-glutamine, and trypsin–EDTA solution were obtained from Gibco Laboratories. Guinea pig liver transglutaminase was purchased from Sigma. Fetal bovine serum was from Hyclone Laboratories, Inc. Endothelial cell growth factor was donated by Dr. Kerri Pratt of the Department of Surgery at Jefferson Medical College of Thomas Jefferson University. The nonpeptidyl transglutaminase inactivator (L682777[1]) was donated by Dr. Andrew M. Stern of Merck Research Laboratories (West Point, PA). Ascites fluid containing a monoclonal antibody (clone P1D6) against human α_5 integrin was obtained from Telios Pharmaceuticals, Inc. (San Diego, CA). Polyclonal antibody against the fibronectin receptor was a gift from Dr. Steven Albelda of the University of Pennsylvania. Silicone oil (DC550) from Dow Corning Corp. was purchased through William K. Nye, Inc. (New Bedford, MA). Light mineral oil was obtained from a pharmacy. [1,4-^{14}C]Putrescine dihydrochloride (108 mCi/mmol and 50 μCi/mL) was from Amersham Corp. (Phenylmethyl)sulfonyl fluoride was from Calbiochem Corp. (La Jolla, CA). Other reagents were from Sigma or Fisher.

Fibronectin Purification and Radiolabeling. Fibronectin was purified from human plasma by gelatin–Sepharose affinity chromatography (Engvall & Ruoslahti, 1977), as previously described (Fellin et al., 1988), and was radiolabeled to a specific activity of 100–700 cpm/ng by a modification (Fellin et al., 1988) of the iodine monochloride method.

Preparation of Endothelial Cells. Primary cultures of HUVEC were prepared from human umbilical cords as described (Sprandio et al., 1988). The cells were maintained in culture with Medium 199 supplemented with 20% fetal calf serum, endothelial cell growth factor (50–100 μg/mL), sodium heparin (50–60 μg/mL), and antibiotics as described (Sprandio et al., 1988). For binding and cross-linking studies with [^{125}I]fibronectin (^{125}I-Fn), confluent cells were washed 3 times with Tris-buffered saline (pH 7.4) and detached with detachment buffer composed of 20 mM Tris-buffered saline (pH 7.4) containing EDTA (10 mM), (phenylmethyl)sulfonyl fluoride (1 mM), benzamidine (5 mM), aprotinin (200 kallikrein inactivating units/ mL), and leupeptin (100 μg/mL). The cells were centrifuged at 1000g for 10 min, washed once in Tris-buffered saline, and resuspended in Medium 199 (for monolayer experiments).

Studies with HUVEC Suspensions or Monolayers. Binding experiments with ^{125}I-Fn and HUVEC suspensions were performed in a manner similar to that described for fibrinogen (Martinez et al., 1989). For monolayer experiments, polystyrene 12-well tissue culture dishes were coated with gelatin, and then ^{125}I-Fn was allowed to adsorb to the gelatin (^{125}I-Fn/gelatin-coated well) as follows: A 1-mL aliquot of 0.2% gelatin solution was added to each well and incubated at room temperature for 2–4 h. The solution was then removed, and the gelatin-coated dish was allowed to dry overnight. Aliquots (500 μL) of Tris-buffered saline containing ^{125}I-Fn (50 μg/mL) were added to each well and incubated at 4 °C for 2 h. The ^{125}I-Fn solution was then removed, and the wells were then washed 5 times with 1-mL aliquots of Tris-buffered saline. Approximately 1–1.5 μg of ^{125}I-Fn remained adsorbed to the gelatin after the washes, and the ^{125}I-Fn/gelatin-coated wells were used in experiments before drying. Endothelial cells ((1–2) × 10^6 cells/mL) were gently layered onto the ^{125}I-Fn/gelatin-coated wells (1 mL/well) and incubated for 3 h at 37 °C, unless indicated otherwise. By this method, (2–3) × 10^5 cells attached to each well (measured using the BCA Protein Assay Reagent Kit from Pierce) and formed a confluent monolayer. For wells containing 20 μM transglutaminase inactivator (L682777), the cells were preincubated with the agent for 10–15 min at room temperature prior to seeding. In experiments where the ^{125}I-Fn was cross-linked by exogenous guinea pig liver tissue transglutaminase, the enzyme was diluted to 10 μg/mL in Medium 199 containing 1% bovine serum albumin added to the ^{125}I-Fn/gelatin matrix and incubated for 1 h at 37 °C. The dishes were then washed 3 times with enzyme-free Medium 199, and HUVEC were seeded as described above. After 3 h of incubation, the conditioned media were collected and centrifuged at 10000g for 5 min, and the supernatants were saved for analysis by SDS–polyacrylamide gel electrophoresis and autoradiography. The cell layers were washed 3 times with Tris-buffered saline, scraped into 100-μL aliquots of Tris-buffered saline, and combined with 100 μL of SDS-reducing buffer. The cell layers and the conditioned media were analyzed by SDS–polyacrylamide gel electrophoresis and autoradiography as described below.

Effect of Conditioned Medium on ^{125}I-Fn/Gelatin-Coated Wells. Endothelial cells were seeded on unlabeled fibronectin-coated dishes for 3 h, and, unless otherwise indicated, the conditioned media were collected, centrifuged at 10000g for 5 min, and incubated on ^{125}I-Fn/gelatin-coated wells for 3 h at 37 °C. The ^{125}I-Fn layer was extracted in reducing buffer and analyzed by SDS–polyacrylamide gel electrophoresis followed by autoradiography. In certain experiments, exogenous guinea pig liver transglutaminase (10–500 ng/mL) was added to fresh Medium 199, and its effect on cross-linking of the gelatin-adsorbed ^{125}I-Fn was compared to the effect of the HUVEC-conditioned medium.

Effect of Spread Cells on Processing of ^{125}I-Fn/Gelatin-Coated Wells. Endothelial cells were seeded on ^{125}I-Fn/gelatin-coated wells in the presence of the transglutaminase inactivator L682777 (20 μM). After 3 h of incubation, when the cells were fully spread, the transglutaminase inactivator was removed by washing the dishes 3 times with fresh incubation medium. At timed intervals after the removal of the inactivator, the culture medium and extracted cell layers were analyzed by SDS–polyacrylamide gel electrophoresis and autoradiography, as described above.

Transglutaminase Assay. The activity of transglutaminase was measured using the filter paper assay (Lorand et al., 1972),

[1] Abbreviations: HUVEC, human umbilical vein endothelial cells; L682777, 1,3,4,5-tetramethyl-2-[(2-oxopropyl)thio]imidazolium chloride; ^{125}I-Fn, human ^{125}I-labeled plasma fibronectin; SDS, sodium dodecyl sulfate; RGDS, Arg-Gly-Asp-Ser.

incubating 25 μL of conditioned medium or extracted cell layer with 0.5 μCi of [^{14}C]putrescine, 0.5 mM cold putrescine, 10 mM $CaCl_2$, and 1 mM DTT in a total volume of 100 μL. The cell layers were washed 3 times with 10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.5, containing pepstatin (5 μg/mL), leupeptin (5 μg/mL), (phenylmethyl)sulfonyl fluoride (0.5 mM), and sodium chloride (150 mM) and scraped into the same buffer containing 1% Triton X-100.

Assay of Cellular Protein and LDH. All samples and the bovine serum albumin standards were in 0.15 M sodium chloride, 0.025 M sodium bicarbonate containing 1% SDS. To 500 μL of samples was added 2 mL of working reagent (prepared according to the instructions). The samples were incubated for 2 h at room temperature, and optical density was read at 660 nm. A standard curve was determined using endothelial cells which had been counted with a hemocytometer. LDH was assayed as described in Sigma Diagnostics Kit, catalog no. 500.

Cell Detachment Assay. Endothelial cells were seeded on unlabeled fibronectin-coated dishes for 3 h in the presence or absence of the transglutaminase inactivator L682777 (20 μM). After 3 h of incubation, the media were removed, the cell layers were washed 3 times with Tris-buffered saline, and the cells were incubated at room temperature in 1 mL of trypsin–EDTA in Hanks' buffer (0.05% trypsin, 0.53 mM EDTA diluted 1:250 in Hanks without Ca or Mg) in a horizontal shaker (2 cm/cycle, 2 cycles/s). After 30 min, the detached cells were combined with FBS 10:1, centrifuged, resuspended in culture media, and counted in a Coulter counter. The remaining attached cells were detached by a 5-min incubation with 0.05% trypsin, 0.53 mM EDTA, and counted in a Coulter counter, and the percentage of attached cells was calculated for each individual well.

SDS–Polyacrylamide Gel Electrophoresis and Autoradiography. Solubilized endothelial cells or cell monolayers were subjected to SDS–polyacrylamide gel electrophoresis as previously described (Barsigian et al., 1991). The cells were suspended in 50–500 μL of 0.02 M Tris, combined with an equal volume of solubilization buffer, and boiled for 2 min. The solubilization buffer consisted of 0.02 M Tris, 2 mM EDTA, 2 mM (phenylmethyl)sulfonyl fluoride, 2 mM *N*-ethylmaleimide, 8 M urea, 4% SDS, and 10% β-mercaptoethanol. The gels consisted of 4% acrylamide stacking and 7.5% acrylamide resolving gels. The proteins were visualized by staining with Coomassie Blue, and autoradiography was performed using standard procedures.

Densitometric Scans. Scanning of autoradiograms was performed, as previously described (Barsigian et al., 1991), using a CAMAC TLC Scanner II on-line with a CAMAC SP4290 integrator. Transmission was measured using a wavelength of 500 nm and a beam width equal to that of the entire lane. The lanes were scanned at 0.4 mm/s, and the intensity of the material with a molecular weight greater than the fibronectin monomer was calculated as a percentage of the total material scanned in each lane.

RESULTS

Previous studies (Conforti et al., 1989; Barsigian et al., 1991) have demonstrated that the binding of fibronectin to endothelial cell suspensions occurs in a time-dependent fashion, requiring 3 h to reach steady state. The interaction of fibronectin with endothelial cell suspensions has also been reported to be mediated via binding to a cellular integrin receptor (Conforti et al., 1989) and to involve calcium-dependent tissue transglutaminase-mediated covalent cross-linking of the bound fibronectin (Barsigian et al., 1991). More recent studies on the time-dependency of the cross-linking process using densitometric scanning of autoradiograms developed from SDS–polyacrylamide gels have revealed that cross-linking of ^{125}I-Fn by HUVEC suspensions was very rapid during the first 1 h of incubation, with 70% of cross-linking occurring during this period and with >90% being cross-linked by 4 h (data not shown). In light of these observations of transglutaminase-mediated processing of soluble fibronectin by HUVEC suspensions, in the present investigation we focused on the potential involvement of tissue transglutaminase in mediating the interaction of endothelial cell monolayers with matrix-immobilized fibronectin.

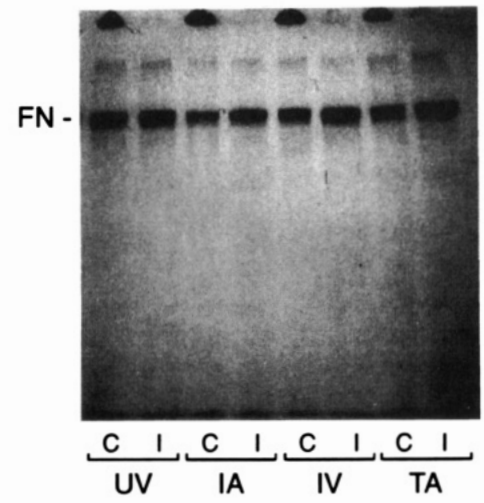

FIGURE 1: Processing of ^{125}I-Fn/gelatin-coated wells by human endothelial cells. Umbilical vein (UV), iliac artery (IA), iliac vein (IV), and thoracic aorta (TA) endothelial cells were seeded onto ^{125}I-Fn/gelatin-coated wells in the presence (I) or absence (C) of 20 μM L682777. After 3 h of incubation at 37 °C, the cell layers were analyzed, under reducing conditions, by SDS–polyacrylamide gel electrophoresis and autoradiography as indicated under Experimental Procedures.

To assess whether endothelial cell monolayers are capable of covalently cross-linking matrix-associated fibronectin, experiments using ^{125}I-Fn/gelatin-coated wells (rather than soluble ^{125}I-Fn) were initiated using endothelial cells of venous or arterial origin. Covalent cross-linking of the ^{125}I-Fn was observed as a decrease in the ^{125}I-Fn monomer associated with the appearance of high molecular weight ^{125}I-Fn-containing complexes at the top of the stacking gel (Figure 1). With each cell type, cross-linking of the ^{125}I-Fn/gelatin matrix was prevented when the cells were seeded onto the matrix in culture medium containing a nonpeptidyl transglutaminase inactivator (Figure 1). These data established the observation that endothelial cells of both venous and arterial origin can cross-link matrix-immobilized fibronectin via a cellular transglutaminase-mediated process.

Because transglutaminase-mediated processing of fibronectin was apparent with a variety of endothelial cell types and since the interaction of fibronectin with HUVEC has been studied previously (Conforti et al., 1989; Barsigian et al., 1991), HUVEC were used for the purpose of analyzing transglutaminase-mediated processing of matrix fibronectin in greater detail. Initially, the time course of cross-linking of subendothelial matrix-associated fibronectin was analyzed. The data illustrated in Figure 2 demonstrate that the multimerization of ^{125}I-Fn reached a substantial level as portrayed by the plot (Figure 2, inset) of the densitometric scan of the autoradiogram which shows that >45% of the gelatin-adsorbed

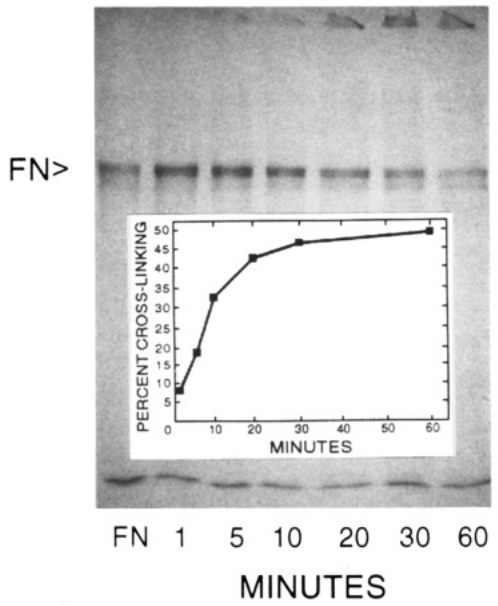

FIGURE 2: Time course of processing of ^{125}I-Fn/gelatin-coated wells by HUVEC monolayers. Aliquots (1 mL) of HUVEC suspended in serum-free Medium 199 (2.5×10^5 cells/mL) were seeded onto ^{125}I-Fn/gelatin-coated wells. At the indicated times, the cell monolayers were analyzed, under reducing conditions, by SDS–polyacrylamide gel electrophoresis and autoradiography as described under Experimental Procedures. Inset: Plot of the densitometric scans of the autoradiogram.

^{125}I-Fn was cross-linked during the first 30 min following initial cell seeding. The cross-linking process then continued at a slower rate during the subsequent incubation period.

Fibronectin binding to endothelial cell suspensions has previously been reported to be mediated by the $\alpha_5\beta_1$ integrin (Conforti et al., 1989). We determined whether this integrin is associated with the covalent cross-linking of subendothelial fibronectin. Under these conditions where $\alpha_5\beta_1$-dependent interaction of the cells with fibronectin was prevented, cell attachment to the matrix was presumably supported by $\alpha_2\beta_1$-dependent interaction with gelatin and/or by integrin-independent interaction of the cells with fibronectin. As seen in Figure 3, neither a monoclonal or polyclonal antibody nor the RGDS tetrapeptide interfered with the covalent cross-linking of the gelatin-adsorbed ^{125}I-Fn, whereas the transglutaminase inactivator L682777 substantially inhibited the cross-linking process. These data suggest that the fibronectin receptor did not directly participate in the transglutaminase-mediated polymerization of fibronectin by endothelial cells.

To differentiate between cross-linking of the gelatin-adsorbed ^{125}I-Fn by cell-associated transglutaminase as opposed to transglutaminase released into the culture medium, experiments using conditioned medium were performed. Figure 4a shows that conditioned medium aspirated from a HUVEC monolayer grown on an nonradiolabeled-Fn/gelatin-coated dish did not contain sufficient transglutaminase to cross-link ^{125}I-Fn present on gelatin-coated dishes. As a positive control, exogenous guinea pig liver transglutaminase (10–500 ng/mL) elicited concentration-dependent cross-linking of the ^{125}I-Fn/gelatin matrix (Figure 4b). Furthermore, the conditioned medium used for Figure 4a was assayed for LDH and for transglutaminase activity and found to be negative for both. These results indicate that tissue transglutaminase was not released from the cells into the medium and that the cross-linking of subendothelial ^{125}I-Fn was mediated by a cell-associated transglutaminase.

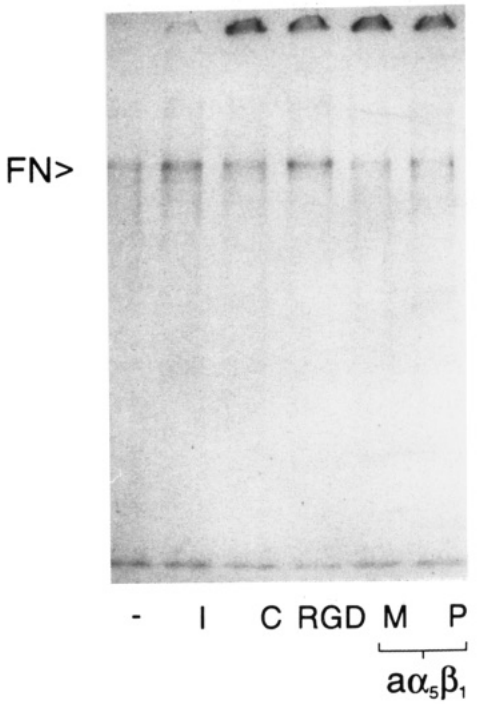

FIGURE 3: Effect of anti-integrins on processing of ^{125}I-Fn by HUVEC monolayers. HUVEC (2×10^5 cells/mL) suspended in serum-free Medium 199 were seeded into ^{125}I-Fn/gelatin-coated wells in the absence (C, control) or presence of 20 μM of the transglutaminase inactivator L682777 (I), 0.5 mM RGDS (RGD), or a monoclonal (M) or polyclonal (P) antibody against $\alpha_5\beta_1$. After 3 h of incubation, the monolayers were analyzed, under reduced conditions, by SDS–polyacrylamide gel electrophoresis and autoradiography as described under Experimental Conditions. The lane marked "–" represents purified ^{125}I-Fn.

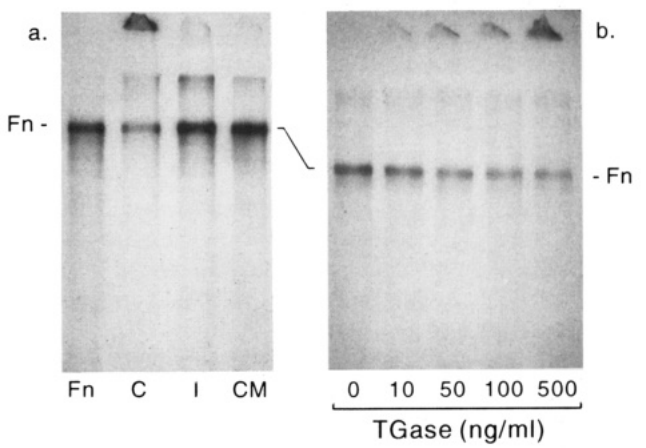

FIGURE 4: Processing of ^{125}I-Fn/gelatin-coated wells by conditioned medium or exogenous transglutaminase. ^{125}I-Fn/gelatin-coated wells were incubated with conditioned medium (panel a) prepared from other HUVEC cultures as described under Experimental Procedures, or with fresh Medium 199 containing various concentrations of exogenous guinea pig liver transglutaminase (panel b). Following 3 h of incubation at 37 °C, the monolayers were analyzed, under reducing conditions, by SDS–polyacrylamide gel electrophoresis and autoradiography. Fn, starting ^{125}I-Fn; C, control wells incubated with HUVEC; I, wells incubated with HUVEC in medium containing 20 μM L682777; CM, wells incubated with conditioned medium; TGase, exogenous guinea pig liver transglutaminase.

To assess whether the transglutaminase-mediated processing of fibronectin by HUVEC monolayers manifested polarity (i.e., was preferentially expressed beneath the cell as opposed to above the cell), experiments were performed in which ^{125}I-Fn was added to serum-free Medium 199 and layered on top of a confluent monolayer of endothelial cells which had been

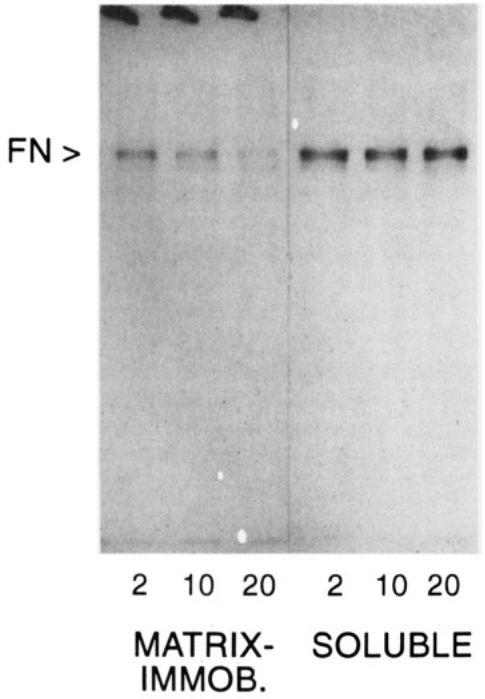

FIGURE 5: Processing of gelatin-adsorbed or soluble ^{125}I-Fn by HUVEC monolayers. HUVEC (2×10^5 cells/mL) were seeded onto ^{125}I-Fn/gelatin-coated wells (matrix-immob) or, alternatively, the cells were seeded onto gelatin-coated dishes and then incubated with culture medium containing 50 nM ^{125}I-Fn (soluble). At the indicated times, the cell monolayers were washed and analyzed, under reducing conditions, by SDS–polyacrylamide gel electrophoresis and autoradiography as described under Experimental Procedures. The numbers 2, 10, and 20 indicate hours of incubation.

seeded on gelatin (without any adsorbed fibronectin). Under these conditions, the soluble ^{125}I-Fn which associated with the apical surface of the endothelial cell monolayer remained in the un-cross-linked form even after a 20-h incubation (Figure 5, soluble), whereas in a parallel incubation using ^{125}I-Fn/gelatin-coated dishes, the ^{125}I-Fn was extensively cross-linked by the adherent cell layer (Figure 5, matrix-immob). These data demonstrate that ^{125}I-Fn added to the medium bathing the apical surface of a confluent HUVEC monolayer did not gain access to the aspect of the cell which expressed transglutaminase activity and that the enzyme appears to be functionally expressed only at the basal surface of the endothelial cell. Whether the enzyme was present on the apical surface of the cell monolayer in a nonfunctional state was not determined.

Experiments were done to assess whether functional expression of enzyme activity was required for cell attachment and spreading to occur. Several experiments were performed, and in each case the cells were fully spread and confluent following a 3-h incubation with the transglutaminase inactivator (data not shown), indicating that transglutaminase activity was not required for initial cell attachment and spreading to occur. Following the 3-h incubation, the transglutaminase inactivator was removed from the cultures. As shown in Figure 6a, by time 0 (i.e., immediately after washing out the transglutaminase inactivator), only a minimal amount of covalent cross-linking of the gelatin-adsorbed ^{125}I-Fn had occurred. However, in the ensuing 40-h period, there was time-de endent cross-linking which reached 30% at 1 h and continued to increase over the next 39 h to reach a maximum of ca. 50% of the total gelatin-adsorbed ^{125}I-Fn. Analysis of the conditioned medium removed from the monolayers at the respective time points revealed that ^{125}I-Fn

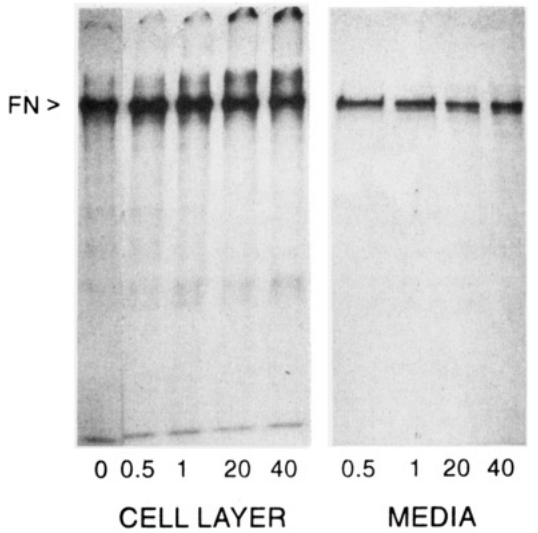

FIGURE 6: Processing of ^{125}I-Fn/gelatin-coated wells by HUVEC monolayers spread in the presence of a transglutaminase inactivator. HUVEC (2×10^5 cells/mL) were seeded onto ^{125}I-Fn/gelatin-coated wells in medium containing 20 μM L682777. After 3 h of incubation at 37 °C, the transglutaminase inactivator was washed out, incubations were continued for the indicated times, and the cell layers and media were analyzed, under reducing conditions, by SDS–polyacrylamide gel electrophoresis and autoradiography as indicated under Experimental Procedures. Serum-free Medium 199 was used for the 0.5- and 1-h incubations, whereas Medium 199 containing 1% serum was used for the 20- and 40-h incubations.

which separated from the substratum was present exclusively as the monomer without any evidence of covalent multimerization, suggesting that tissue transglutaminase-mediated cross-linking may have stabilized the association of fibronectin with the matrix.

To address this question using a different approach, experiments were performed to determine the effect of tissue transglutaminase-mediated covalent cross-linking on the rate of release of ^{125}I-Fn from the matrix into the culture medium. Under control conditions (i.e., when the endothelial cells were incubated in the absence of the transglutaminase inactivator and therefore capable of cross-linking ^{125}I-Fn), the release of monomeric ^{125}I-Fn into the medium increased over the 40-h evaluation period to reach a level of ca. 40% of the total ^{125}I-Fn which was initially adsorbed to the matrix (Figure 7). It is important to note that cross-linked ^{125}I-Fn was not present in the medium at any time during the incubation (Figure 6). When cross-linking of fibronectin was prevented by incubation of the cells in the presence of the transglutaminase inactivator, the extent of release increased until ca. 75% of the ^{125}I-Fn was free in the medium at 40 h (Figure 7). These data demonstrate that cross-linking of subendothelial fibronectin by endogenous endothelial cell-associated transglutaminase has a stabilizing effect on the association of the fibronectin with the underlying extracellular matrix. Because the endothelial cell monolayers usually do not cross-link all of the matrix fibronectin within the time frame of our experiments, we artificially induced total cross-linking of the gelatin-adsorbed ^{125}I-Fn by adding exogenous guinea pig liver transglutaminase to the matrix prior to the seeding of the endothelial cells. Under these conditions, the release of ^{125}I-Fn into the culture medium was drastically reduced so that even at 40 h <10% of the initially adsorbed ^{125}I-Fn was present in the medium (Figure 7), thus corroborating the observation made with the endogenous endothelial cell-associated tissue transglutaminase.

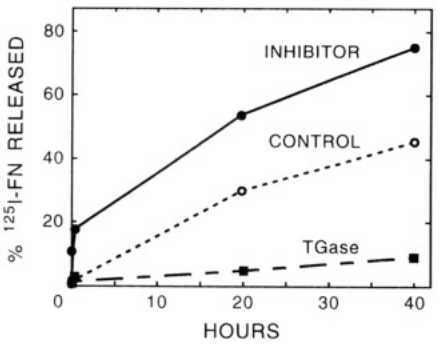

FIGURE 7: Release of subendothelial ^{125}I-Fn into the culture medium of HUVEC monolayers. HUVEC (2×10^5 cells/mL) were seeded onto ^{125}I-Fn/gelatin-coated wells in the presence (●) or absence (○) of 20 μM L682777. A third group of cells was seeded on ^{125}I-Fn/gelatin-coated wells that had been preexposed to 10 μg/mL of guinea pig liver transglutaminase for 1 h at 37 °C (■) as described under Experimental Procedures. At the indicated times, aliquots of media were removed and the released ^{125}I-Fn was counted in a γ-counter. The matrix-adherent ^{125}I-Fn was solubilized in 10 M NaOH overnight, and the percent of total ^{125}I-Fn released was calculated as ^{125}I-Fn in media divided by ^{125}I-Fn in media plus matrix.

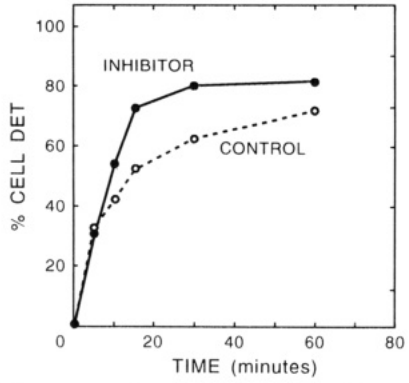

FIGURE 8: Time course of trypsin-induced detachment of HUVEC from a fibronectin/gelatin matrix. HUVEC (2×10^5 cells/mL) were incubated on matrix-immobilized Fn in the presence (●) or absence (○) of transglutaminase inactivator as described under Experimental Procedures. After 3 h of incubation, the monolayers were washed 3 times and incubated in trypsin–EDTA in Hanks' buffer. At the indicated times, the detached cells were counted and expressed as a percent of the total number of cells which were initially attached.

We also determined whether endothelial cell-associated transglutaminase-mediated cross-linking of subendothelial fibronectin influenced the sensitivity of the cells to proteolytic detachment from the underlying matrix. As seen in Figure 8, endothelial cells which had been seeded in the presence of the transglutaminase inactivator detached earlier from the Fn/gelatin matrix than did the control cells. Only 15 min was required for detachment of ca. 75% of cells incubated with the transglutaminase inactivator, whereas 60 min was required for detachment of 75% of control cells. These data demonstrate that the extracellular expression of endothelial cell-associated tissue transglutaminase plays an important role in reinforcing endothelial cell adherence to the matrix.

The preceding experiments demonstrate that cross-linking of subendothelial fibronectin by endothelial cell-associated tissue transglutaminase stabilizes the association of the fibronectin (Figures 6 and 7) as well as the endothelial cells (Figure 8) with the underlying extracellular matrix. Since cross-linked fibronectin was not spontaneously released into the medium (Figure 6), we hypothesized that, in like manner, the resistance of the cells to trypsin-induced detachment may have been due to an increased resistance of cross-linked

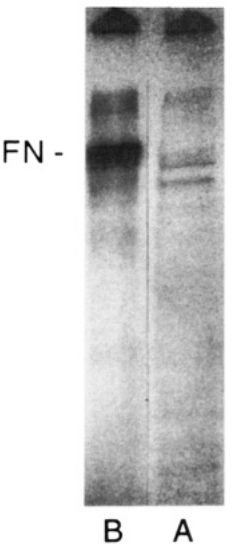

FIGURE 9: Molecular integrity of subendothelial ^{125}I-Fn following trypsin-induced cell detachment. HUVEC were seeded onto ^{125}I-Fn/gelatin-coated wells. After 3 h of incubation at 37 °C, wells were either processed for SDS–PAGE and autoradiography (B) or incubated with trypsin for 2 h, as described under Experimental Procedures, and then processed for SDS–PAGE and autoradiography (A).

fibronectin to trypsin-induced separation from the matrix and/or increased resistance to proteolysis. As shown in Figure 9, the ^{125}I-Fn which remained adherent to the culture dish following a 2-h incubation with trypsin (a procedure which detached all the cells) was present predominantly as highly cross-linked ^{125}I-Fn on top of the stacking gel, with most of the monomeric fibronectin having undergone proteolysis (Figure 9, lane A). In contrast, intact cell monolayers which were scraped and analyzed without trypsin treatment exhibited not only the high molecular weight ^{125}I-Fn but also a substantial amount of monomeric ^{125}I-Fn (Figure 9, lane B). Similar results were obtained after a 30-min incubation (data not shown). These data indicate that the covalent cross-linking of the ^{125}I-Fn by the endothelial cell-associated tissue transglutaminase contributed resistance to detachment from the matrix by trypsin.

DISCUSSION

The major finding of the current investigation relates to the processing of subendothelial matrix-immobilized fibronectin by cultured human endothelial cells. We demonstrate that endothelial cell monolayers, as opposed to endothelial cell suspensions, can covalently cross-link substrate-immobilized fibronectin via a process mediated by an endothelial cell-associated tissue transglutaminase and that this phenomenon occurs with endothelial cells of both arterial and venous origin. In contrast, when fibronectin was added to the overlying culture medium of confluent HUVEC monolayers, it failed to be cross-linked into high molecular weight multimers, indicating that the cross-linking process is topographically localized to the basal aspect of the cell, a region which mediates the matrix assembly of fibronectin (Kowalczyk et al., 1990; Kowalczyk & McKeown-Longo, 1992). This may be because transglutaminase is not expressed at the apical surface of the cell, ^{125}I-Fn in solution is not an efficient substrate for substrate-adherent endothelial cell-associated transglutaminase, or the ^{125}I-Fn which was added to the medium failed to cross the HUVEC monolayer and was, therefore, unavailable for cross-linking by the basally expressed tissue transglutaminase. The latter explanation is consistent with studies demonstrating

that fibronectin does not pass through confluent endothelial cell monolayers, as has been reported to occur with fibroblasts (Kowalczyk et al., 1990).

The binding of fibronectin to endothelial cells in suspension has been reported to involve RGD-dependent interaction with the endothelial cell integrin $\alpha_5\beta_1$ (Conforti et al., 1989). More recently, it has been shown (Lampugnani et al., 1991) that the integrin heterodimers $\alpha_2\beta_1$ and $\alpha_5\beta_1$ are present at areas of cell–cell contact, and the ligands for these integrins—laminin, type IV collagen, and fibronectin—play a fundamental role in maintaining the continuity of the monolayer. Moreover, interference with the $\alpha_5\beta_1$ integrin induces discontinuities in the monolayer, increasing the permeability of the monolayer to macromolecules (Lampugnani et al., 1991). For these reasons, we investigated the potential involvement of the $\alpha_5\beta_1$ integrin in the transglutaminase-mediated covalent cross-linking of subendothelial fibronectin by endothelial cell monolayers. On the basis of the observation that the cross-linking of fibronectin by HUVEC was not affected by antibodies against the fibronectin receptor or by the Arg-Gly-Asp-Ser tetrapeptide, we conclude that the $\alpha_5\beta_1$ fibronectin receptor (Hynes, 1990; Ruoslahti, 1988; Albelda et al., 1989; Yamada, 1991) did not directly participate in the transglutaminase-mediated cross-linking process. However, the potential involvement of RGD-independent integrins in this phenomenon was not investigated.

The processing of the gelatin-adsorbed ^{125}I-Fn by the HUVEC monolayers appeared to involve two distinct phases—an initial rapid phase which occurred during the 30-min period following the initial seeding of cells and a more sluggish phase which was operative for the entire duration of the incubation. The covalent cross-linking of subendothelial ^{125}I-Fn/gelatin also increased, in a time-dependent fashion, following initial cell attachment and spreading under conditions where cross-linking was temporarily prevented by a 3-h incubation in medium containing the transglutaminase inactivator L682777. These findings indicate that the expression of transglutaminase activity by the endothelial cells and the covalent cross-linking of extracellular matrix-associated fibronectin were not required for the attachment and spreading of the cells and that tissue transglutaminase continues to be functionally expressed after the establishment of an intact cellular monolayer. During the postspreading stage, the covalent cross-linking of fibronectin eventually reached a steady state when ca. 50% of the gelatin-adsorbed ^{125}I-Fn was polymerized. Thus, transglutaminase expression by endothelial cells in culture is a continuous process which may be operative but not essential for the initial cell attachment and spreading and which continues during postspreading remodeling of the matrix.

The biological implications of cross-linking of matrix fibronectin by endothelial cells are unclear. However, recent studies have shown that the induction of tissue transglutaminase synthesis is associated with morphological changes and increased cell adhesiveness (Byrd & Licthi, 1987; Gentile et al., 1992). In a recent study (Gentile et al., 1992), the transfected cells took on a more flattened appearance and were more resistant to trypsin-induced detachment from culture dishes than were nontransfected transglutaminase-negative cells. However, the role of matrix cross-linking by the transglutaminase-transfected cells was not investigated. In our present study, we corroborate the findings of these investigators (Gentile et al., 1992) that transglutaminase expression by cells increases the resistance of cells to trypsin-induced detachment from the matrix, and we correlate this finding with the extracellular expression of endothelial cell-associated transglutaminase as evidenced by covalent cross-linking of the subendothelial fibronectin. Our study also shows that tissue transglutaminase-mediated cross-linking of fibronectin at the basal surface of endothelial cell monolayers inhibits spontaneous as well as trypsin-induced separation of the multimerized fibronectin from the cell layer into the medium. Such processing of matrix proteins by cell-associated tissue transglutaminase may serve to firmly anchor cells to the extracellular matrix, conferring upon them resistance to detachment by proteolytic enzymes, by high shear stress, or by other pathological forces acting to disrupt the integrity of the endothelium. Whether the reinforced attachment of the cells to the matrix is due to covalent cross-linking of cellular components to the matrix proteins or merely to a greater affinity of the cells for the transglutaminase-induced polymerized form of the matrix-associated fibronectin is currently under investigation.

The functional expression of tissue transglutaminase at the basal aspect of the endothelial cell monolayer may also serve to stabilize the matrix-associated fibronectin by virtue of the formation of the highly cross-linked multimers. In support of this finding is the observation that fibrin clots which are extensively cross-linked by factor XIIIa are known to be more resistant to plasmin-mediated lysis than are less highly cross-linked clots (Francis & Marder, 1988). Our experiments demonstrate that only the monomeric form of ^{125}I-Fn separated from the matrix into the medium of confluent HUVEC monolayers and that the extent of release decreased as the amount of cross-linking increased. Moreover, the multimerized fibronectin was never found in the culture medium, as it remained firmly attached to the gelatin-coated dishes. Moreover, the cross-linked fibronectin was resistant to trypsin-induced separation from the matrix under conditions where the monomeric form of fibronectin was readily released and degraded. This observation suggests that transglutaminase-mediated covalent cross-linking of subendothelial fibronectin may render the cross-linked fibronectin a less suitable substrate for trypsin, much like cross-linked fibrin is for plasmin (Francis & Marder, 1988). It is also possible that the covalent processing of matrix proteins by cell-associated tissue transglutaminase may confer upon the matrix a resistance to endogenous cell-associated proteolytic activity mediated by the plasminogen activator/plasmin system (Vassalli et al., 1991). Such resistance to natural proteolytic enzymes may play an important role in the biochemistry of such processes as cell migration and tumor metastasis.

Our results resemble some aspects of the multimerization of fibronectin by fibroblasts (McDonald, 1988; Mosher et al., 1991). In this system, the dimeric fibronectin molecules interact with cell surface receptors and are subsequently assembled into an insoluble fibrillar pericellular matrix (McDonald, 1988; Mosher et al., 1991; Ruoslahti et al., 1981; Hedman et al., 1979; Singer, 1979). The fibrils are composed of fibronectin which is multimerized by the cells via formation of interchain disulfide bonds (Carter & Hakomori, 1981; Choi & Hynes, 1979; Hynes & Destree, 1977; McKeown-Longo & Mosher, 1984). The N-terminal region of the fibronectin molecule plays an essential role in the polymerization process (Quade & McDonald, 1988; Schwarzbauer, 1991). If exogenous factor XIIIa is added to the fibroblast cultures, however, cross-linking and transfer of fibronectin into the insoluble pericellular matrix are markedly increased (Barry & Mosher, 1988; Barry & Mosher, 1989). Factor XIIIa can also cross-link fibronectin which has already undergone

multimerization via disulfide bond formation (Barry & Mosher, 1988; Barry & Mosher, 1989), and the formation of fibrillar fibronectin occurs not only *in vitro* but also *in vivo* in the granulation tissue of wounds (Singer et al., 1984). Our results with endothelial cells differ considerably from those with fibroblasts in that it is the endogenous transglutaminase of the cell that cross-links the fibronectin. Thus, the subendothelium, which is not normally exposed to plasma factor XIII, may undergo cross-linking as the result of basal expression of endothelial cell tissue transglutaminase, which may stabilize the matrix and the overlying endothelial cell monolayer.

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