Fibronectin Fragments Promote Human Retinal Endothelial Cell Adhesion and Proliferation and ERK Activation through $\alpha_5\beta_1$ Integrin and PI 3-Kinase

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PURPOSE. Extracellular matrix degradation is associated with neovascularization in diabetic retinas. Fibronectin fragments (Fn-fs) are generated during vascular remodeling. The effects of cellular fibronectin (Fn) and selected Fn-fs on adhesion, proliferation, and signal transduction in human retinal endothelial cells (HRECs) were characterized.

METHODS. Relative quantitative RT-PCR, flow cytometry, and immunocytochemistry determined integrin expression on HRECs. Adhesion was evaluated by coating plastic with Fn or Fn-fs of 45, 70, 110, or 120 kDa, and MTT conversion was used to measure proliferation and survival. Peptide inhibitors and blocking antibodies determined adhesive sites and integrins used for adhesion. Pharmacologic inhibitors and Western analyses were used to evaluate intracellular signaling.

RESULTS. HRECs produced significant levels of $\alpha_5$, $\alpha_4$, $\alpha_2$, $\alpha_v$, $\beta_1$, $\beta_3$, and $\beta_5$ integrin subunit mRNA. Flow cytometry of surface integrin expression revealed high levels of $\alpha_5$, $\alpha_4$, and $\beta_1$ and lower levels of $\alpha_2$, $\alpha_v$, $\beta_3$, and $\beta_5$. These results were confirmed by immunocytochemistry. For adhesion to Fn and Fn-fs, the $\alpha_5\beta_1$ integrin was essential. Pharmacologic inhibitors of PI 3-kinase blocked adhesion to Fn and Fn-fs, whereas the mitogen-activated protein (MAP) kinase kinase (MEK) inhibitor PD98059 blocked phosphorylation. The 110- and 120-kDa Fn-fs showed a concentration-dependent increase in proliferation, whereas 500 ng of the 70 kDa Fn-f-induced proliferation. Addition of III1-C, a matrix assembly domain, increased the proliferative effect of these Fn-fs.

CONCLUSIONS. Fn and its Fn-fs modulate HREC adhesion and proliferation through signal-transduction pathways involving coupling of the $\alpha_5\beta_1$ integrin through PI 3-kinase. Mitogenic signals for endothelial cells from degraded extracellular matrix may contribute to the development of diabetic retinopathy. (Invest Ophthalmol Vis Sci. 2003;44:1704–1715) DOI:10.1167/iovs.02-0775

Diabetic retinopathy is the leading cause of adult blindness in the United States. Proliferative diabetic retinopathy is associated with aberrant endothelial cell proliferation, neovascularization, vitreous hemorrhage, and traction detachment. Increased amounts of aberrant extracellular matrix (ECM) observed in retinal vessels of diabetic patients may contribute to the endothelial cell dysfunction that is characteristic of this disease. Retinal vessels of diabetic patients contain increased amounts of fibronectin (Fn)5,4 and in vitro exposure of retinal endothelial cells to high glucose levels increases secretion of Fn.5 Retinas of patients with diabetic retinopathy are positive for the splice variant ED (extra domain)-B Fn, a marker of angiogenesis,8 suggesting a role for locally synthesized Fn in neovascularization.

Fn is a multifunctional glycoprotein found in plasma and ECM that regulates cellular adhesion, migration, oncogenic transformation, wound healing, and hemostasis.7 It exists as a 450-kDa dimer with subunits joined by a pair of disulide bonds located near the carboxyl termini. The diverse biological activities attributed to Fn have been localized to specific regions of the molecule (Fig. 1). These regions were identified by their binding affinity for specific molecules such as fibrin, factor XIIIa, gelatin-collagen, and heparin. In addition to binding domains, functional domains, such as the matrix assembly domain located near the amino terminus, and the cell-binding domain, which spans type III repeats 8 to 10, have also been extensively characterized.8,9 Fn is highly susceptible to proteolysis, often generating fragments (Fn-fs) with greater or different biological activity than the parent protein.10–12 Degradation of Fn occurs in the vicinity of cells undergoing neoplastic transformation, possibly due to expression of proteases by cancer cells.13 We have demonstrated that latent matrix metalloproteinase (MMP)-2, secreted by human retinal endothelial cells (HRECs), exists as a complex with the 30-kDa N-terminal portion of Fn and that binding of this fragment to latent MMP-2 inhibits the activation of this latent protease.14 This suggests the active involvement of Fn and Fn-fs in regulating proteolytic enzymes in diabetic neovascularization. We have shown that the 120-kDa N-terminal Fn-f is a potent mitogen for HRECs, inducing greater proliferation than fibroblast growth factor (FGF)-2.15 Fn-fs induce cell proliferation, cause release of cytokines from vascular cells,12,14 and modulate adhesion, spreading, and migration of vascular endothelial cells.14 These studies suggest that the Fn and Fn-fs present in microvascular basement membranes may modulate neovascularization in the diabetic retina.

Endothelial cells are anchorage dependent and require both adhesion to the ECM and growth factor stimulation for survival, growth, and differentiation. Many of the adhesive contacts are mediated through integrins, heterodimeric receptor molecules formed by ion-dependent, noncovalent binding of one $\alpha$ and one $\beta$ transmembrane glycoprotein subunit.16 The extracellular domain of each subunit binds to several ligands including the ECM proteins Fn, vitronectin (Vn), and collagen.
Similar to growth factor activation, engagement of integrins initiates kinase cascades that activate multiple growth-associated kinases including focal adhesion kinase (FAK), phosphoinositide 3-OH kinase (PI 3-kinase), src, and raf, and the mitogen-activated protein (MAP) kinase kinase (MEK). Conversely, lack of attachment to the ECM through integrins induces anoikis (cell death by detachment).

In this study, we determined the expression of integrin subunits on HRECs by relative quantitative RT-PCR, flow cytometry, and immunocytochemistry. We compared the effect of Fn and key Fn-fs on cell adhesion and cellular signaling pathways associated with adhesion, proliferation, and cell survival. Our data indicate that the Fn-fs examined bind to the same integrin (αβ3) as the parent protein and transduce signals to activate extracellular signal-regulated kinase (ERK) through a PI 3-kinase-dependent pathway. These data suggest that the interaction of HRECs with Fn and its proteolytic fragments initiate common intracellular signaling events that contribute to adhesion and proliferation.

**METHODS**

**Materials and Reagents**

Cell culture reagents were obtained from Invitrogen (Carlsbad, CA), except for insulin-transferrin-selenium and endothelial cell growth supplement, which were purchased from Sigma-Aldrich (St. Louis, MO). Wortmannin, LY294002, U0126, and PD98059 were purchased from Calbiochem (La Jolla, CA); purified 70- and 45-kDa Fn-fs from Sigma-Aldrich; and cert-cell growth factor (acetylated LDL) from Beckman-Coulter (Hialeah, FL) within 36 hours of death (n = 3 donors). Human retinal endothelial cells were prepared and maintained as previously described, and cells between passages 3 and 5 were used for the present study. The identity of HRECs is typically validated by demonstrating endothelial cell incorporation of fluorescein-labeled acetylated LDL.

**Relative Quantitative RT-PCR**

Relative quantitative RT-PCR was performed as previously described. Total RNA was isolated from fresh cryopreserved tissue (TRizol; GibcoBRL, Grand Island, NY), according to the manufacturer’s instructions. Reverse transcription was performed with 2 μg of RNA and reverse transcriptase (Superscript MMLV RNase H– Invitrogen), according to the manufacturer’s instructions. PCR was performed on the cDNA with previously described primers or g-actin (GAPDH) as an internal control. Data were normalized to GAPDH.

**Flow Cytometry**

A nonenzymatic dissociation was used to remove HRECs from culture dishes, to preserve the integrity of the cell surface molecules. Cells were washed four times with Ca2+/Mg2+-free PBS and were then incubated for 15 minutes in 2 mM EDTA in Ca2+/Mg2+-free PBS at 37°C and washed four times with PBS. Cells were incubated for 30 minutes on ice with individual integrin antibody diluted in PBS containing 1% bovine serum albumin. Cells were washed and then incubated with the appropriate fluorescein-labeled secondary antibody for 30 minutes on ice. Nonimmune species and isotype-matched antibodies were used as negative controls. Appropriate FITC-conjugated secondary antibodies were added and incubated. After a final wash, cells were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer. Finally, samples were analyzed for integrin expression following flow cytometry.

**Immunocytochemistry**

HRECs were grown to 60% confluence (104 cells/cm2) in eight-well chamber slides (Laboratory-Tek, Naperville, IL) coated with 0.5 μg/ml anti-rabbit, or anti-goat fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were from Sigma-Aldrich. Recombinant human III-C Fn-f was a kind gift from Alex Morla (University of Chicago, Chicago, IL) and additional III-C was obtained from Sigma-Aldrich.
The cells were washed with PBS, fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, washed with PBS, and incubated with 10% normal blocking serum in PBS for 30 minutes to suppress nonspecific binding of IgG. The blocking serum used was from the identical species in which secondary antibody was raised. Cells were then incubated with the primary antibody for 1 hour at 37°C and with the appropriate FITC-conjugated secondary antibody, diluted with 1.5% blocking serum in PBS for 30 minutes at 37°C. Nonimmune species and isotype-matched antibodies were used as negative controls. The cells were washed, mounted in glycerol/PBS, and photographed with a fluorescence microscope (Axiophot; Carl Zeiss; Thornwood, NY).

**Evaluation of Fn-fs’ Purity and Binding of Fn and Fn-fs to Tissue Culture Plastic**

Fn and Fn-fs were obtained from three separate suppliers, because the fragments were not available from a single supplier. However, all

<table>
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<th>Subunit</th>
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Data were normalized to glyceraldehyde phosphate dehydrogenase levels and are expressed as arbitrary units.

**Figure 2.** Surface integrin expression in HRECs. Flow cytometry was used to determine the surface expression of integrin subunits. (A) A representative profile for β1. (B) Quantification of three separate experiments.
fragments were purified by the manufacturer, by high-performance liquid chromatography, and were then reconstituted according to manufacturer’s instructions and stored at −80°C in single-use aliquots. With each purchase of fragments we analyzed each fragment by SDS-PAGE and confirmed that each sample consisted of a single band by silver staining analysis.

Tissue culture dishes were coated overnight (4°C with Fn or the Fn-fs (0.1–10 μg/mL in PBS). Parallel plates were also coated with 100 μg/mL of each Fn-f and washed twice with PBS. Protein attachment was detected by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL), to ensure that each Fn-f bound to the plastic culture wells with the same avidity. Plates for adhesion and proliferation assays were blocked for 1 hour with 2% BSA in PBS before use.

**Adhesion of HRECs**

HRECs were incubated for 2 hours in 96-well culture plates coated overnight with cellular Fn and the 45-, 70-, 110-, or 120-kDa Fn-fs diluted in PBS, as described earlier. HRECs were serum-starved for 24 hours, dissociated in 0.5% trypsin-EDTA, and washed two times in 1% BSA in 1:1 DMEM/F-12. Cells were incubated for 1 hour in 2% BSA in Dulbecco’s modified Eagle’s medium (DMEM/F-12 (1:1) before assay and then plated (5 × 10^5/well). They were allowed to adhere for 2 hours at 37°C, 5% CO2 after preincubation for 10 minutes with RGDS, RGES, or III1-C peptide. Nonadherent cells were removed by washing twice with PBS. Concentrations of Fn-fs were chosen at which a dose-dependent effect was observed, rather than selecting the optimal concentration, to increase the likelihood of observing the effects of the added peptides. HREC proliferation was measured by a modified MTT assay, which measures the ability of live cells to use thiozolyl blue and convert it to dark blue formazan. Complete growth medium containing 0.5 mg/mL MTT was added, and MTT conversion was measured using a microplate spectrophotometer (absorbance, A550–A690). For antibody-blocking experiments, HRECs were processed as described earlier and preincubated with antibody to β1, β2, β3, α, β1, or α3, β13 diluted to 10 μg/mL (except for α3,β3, which was used at 100 μg/mL) in PBS containing 2% BSA for 10 minutes before being plated. Control cells were incubated with nonimmune and isotype matched species antibodies. In experiments in which kinase inhibitors were used, cells were incubated for 30 minutes in the presence of the inhibitors, and control cells were incubated in 0.01% dimethyl sulfoxide (DMSO) for experiments with wortmannin, LY294002, U0126, and PD98059.

**Kinase Activation Assays**

Cells were serum-starved for 24 hours (1:1 DMEM/F-12), dissociated as described earlier, allowed to recover for 1 hour, and incubated in the presence of inhibitors (PD98059, LY294002, wortmannin or 0.01% DMSO as a control) for 30 minutes before stimulation. Cells were plated on Fn- and Fn-f-coated cell culture dishes (as described for adhesion assays) and allowed to adhere for 2 hours. Nonadherent cells were collected by centrifugation (800g) at 4°C. The assay was terminated with the addition of lysis buffer containing protease and phosphatase inhibitors (1% Triton X-100, 10 μg/mL aprotinin, 20 μM leupeptin, 1 μM E-64, 1 mM NaF, 200 μM sodium pervanadate, 1 mM dithiothreitol, 5 mM EDTA, and 25 mM Tris [pH 6.8]) and frozen at −20°C until assayed. Electrophoresis was performed according to the method described by Laemmli. Proteins were fractionated on 10% polyacrylamide gels. Parallel gels were stained with Coomassie blue to verify loading, sample integrity, and protein separation. Proteins were transferred for 2 hours (50 V) from acrylamide gels to polyvinyl difluoride (PVDF) membranes for immunodetection. Membranes were blocked for 1 hour with 5% nonfat powdered milk in TBS (25 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20 [pH 7.3]) and probed at room temperature (phospho-ERK, 1:1000 in TBS, 6 hours or total ERK 1:40,000 in TBS for 1 hour). HRP-conjugated anti-rabbit or anti-mouse was used for detection at a dilution of 1:1000. Secondary antibody incubations were for 1 hour, and membranes were washed three times in TBS between antibody incubations. Peroxidase activity was detected using chemiluminescence with 1- to 5-minute exposure times. Densitometry was performed on the film (Scion Image, Frederick, MD). Average background density was subtracted, and optical densities were plotted on computer (Origin; Microcal, Northampton, MA).

**HREC Proliferation Measured by MTT Conversion**

The effect of Fn-fs on cell proliferation was measured by MTT conversion. Proliferation was measured using 10^5 cells/well seeded in 96-well microtiter plates coated with proteins as indicated above. HRECs were incubated for 96 hours in DMEM/F-12 (1:1) supplemented with insulin-transferrin-selenium and 1% endothelial cell growth supplement. Next, 10 μL of MTT (5 mg/mL) cells was added to each well (0.5 mg/mL, final concentration). Plates were returned to the incubator after addition of MTT. The assay was terminated by aspiration of the medium with beveled needle, and MTT was solubilized in 100 μL isopropanol. MTT conversion was measured with a microplate spectrophotometer (A550–A690).

**Statistical Analysis**

Data were analyzed by ANOVA on computer (Origin Microcal). Each concentration was compared with control levels and the difference considered significant at P < 0.05.

**RESULTS**

A schematic representation of Fn and the regions contained in each of the five Fn-f examined is shown in Figure 1. The 70-kDa Fn-f contains the amino terminal fibrin-heparin and the colla-
levels on the cell surface. Integrin subunit expression. Figure 2 details the cumulative results of whether mRNA levels were representative of the surface integrin expression. Flow cytometry was then used to determine the highest levels, consistent with the role of this integrin dimer in vascular beds. Flow cytometry was then used to determine the highest levels, consistent with the role of this integrin dimer in vascular beds. Flow cytometry was then used to determine whether mRNA levels were representative of the surface integrin expression. Figure 2 details the cumulative results of flow cytometry analysis of surface integrin expression by HRECs. Integrins containing subunits αα, ββ, and ββ were present at high levels on the cell surface. Integrin subunit αα was also expressed, as was αα, although to a lesser degree than either αα or αα. The ββ subunit was also highly expressed, and to a lesser degree ββ and ββ. All the integrins detected by RT-PCR were also detected by flow cytometry, except αα mRNA that was not detected by RT-PCR but was detected by flow cytometry and immunocytochemistry. The immunocytochemical localization of the αα and ββ integrin subunits on HRECs is shown in Figures 3A and 3B, respectively. Integrin subunits αα and ββ were also immunolocalized and reacted with similar intensity, whereas integrin subunits αα, ββ, and ββ reacted with less intensity (not shown). Negative controls did not stain (not shown).

Integrin Expression of HRECs

Relative RT-PCR and flow cytometry were used to determine which integrins were expressed by HRECs and available to bind Fn. HRECs produced significant levels of αα, αα, αα, ββ, ββ, and ββ integrin subunit mRNA. Table 1 details the mRNA expression for integrin subunits in HRECs, normalized to GAPDH. The αα and ββ integrin mRNAs were expressed at the highest levels, consistent with the role of this integrin dimer in endothelial cell adhesion to various ECM molecules in other vascular beds. Flow cytometry was then used to determine whether mRNA levels were representative of the surface integrin expression. Figure 2 details the cumulative results of flow cytometry analysis of surface integrin expression by HRECs. Integrins containing subunits αα or αα were present at high levels on the cell surface. Integrin subunit αα was also expressed, as was αα, although to a lesser degree than either αα or αα. The ββ subunit was also highly expressed, and to a lesser degree ββ and ββ. All the integrins detected by RT-PCR were also detected by flow cytometry, except αα mRNA that was not detected by RT-PCR but was detected by flow cytometry and immunocytochemistry. The immunocytochemical localization of the αα and ββ integrin subunits on HRECs is shown in Figures 3A and 3B, respectively. Integrin subunits αα and ββ were also immunolocalized and reacted with similar intensity, whereas integrin subunits αα, αα, ββ, and ββ reacted with less intensity (not shown). Negative controls did not stain (not shown).

HREC Adhesion to Fn and Fn-fs

Adhesion was supported on plates coated with cellular (c)-Fn and the 70-, 110-, and 120-kDa Fn-fs (Fig. 4). The 110-kDa fragment supported the greatest adhesion up to 10 μg/mL, whereas the 70- and 120-kDa Fn-fs and c-Fn supported similar levels of adhesion but less than the 110-kDa Fn-Fn. As expected, the 70-kDa fragment was not as potent at promoting Fn adhesion as were the Fn-fs containing the cell-binding domain. Preincubation with the matrix assembly–promoting III1-C peptide23 potentiated adhesion to all Fn-fs and to c-Fn (Fig. 4). The 5-kDa fragment did not support adhesion, even in the presence of the III1-C peptide, and it was not capable of blocking adhesion to the other fragments tested or to c-Fn (not shown). In addition, adhesion to all substrates was blocked by preincubation with the RGDS peptide, which un couples RGD-dependent integrin binding, but not the control peptide RGES (Fig. 4). Adhesion to the 110-kDa Fn-Fn was only partially blocked by RGDS, indicating either increased affinity of this fragment for the integrin or the presence of an alternate cell-binding domain or integrin partner. Adhesion to the 70-kDa fragment, which does not contain the cell-binding domain, was also blocked by RGDS. It has been suggested, that this is due to cross-competition between the RGD sequence and a second integrin-binding site in the N-terminal fragment.24 These data indicate that HRECs adhere to Fn and Fn-fs through integrins and require the presence of...
the RGD sequence, regardless of whether binding occurs directly at the cell-binding domain.

Inhibition of HREC Adhesion to Fn and Fn-fs by Integrin Antibodies

Expression data were used to select candidate integrins for antibody blocking experiments. We first tested blocking antibodies against the $\alpha_{\text{IIb}}\beta_3$ subunits that were expressed (Fig. 5). Anti-$\alpha_{\text{IIb}}$ blocked adhesion to the 70- (Fig. 5A), 110- (Fig. 5B), and 120-kDa (Fig. 5C) Fn-fs and to c-Fn (Fig. 5D). Blocking antibodies against $\alpha_5$ and $\beta_5$ integrins did not block adhesion to any of the proteins tested.

The $\beta_1$ subunit is capable of forming dimers with several $\alpha$-subunits. The $\alpha_\beta_1$ dimer is relatively selective for Fn, although Fn can also bind the vitronectin-selective $\alpha_\beta_3$ integrin under certain experimental conditions. Anti-$\alpha_\beta_1$ did not block adhesion to any of the fragments tested (data not shown). Preincubation with anti-$\alpha_\beta_1$ (10 ng/mL) blocked adhesion to the 70,-, 110-, and 120-kDa Fn-fs and Fn-f (Fig. 6). Although some promiscuity exists in integrin-ECM interactions, preincubation with anti-$\alpha_\beta_1$ (up to 100 ng/mL) did not alter adhesion to any of the fragments tested (Fig. 6).

PI 3-Kinase Activity in Adhesion

We next examined the intracellular signaling pathways activated by integrin engagement to promote adhesion. Cells were pretreated with wortmannin or LY294002 (inhibitors of PI 3-kinase) and allowed to adhere to each Fn-f or c-Fn for 2 hours. Wortmannin and LY294002 partially blocked adhesion to all fragments, indicating a role for PI 3-kinase in HREC adhesion (Fig. 7). Wortmannin (100 nM) was more potent than LY294002 (50 μM) in inhibiting adhesion, but considerable variability existed between donors in the degree of observed inhibition with these compounds. When cells were pretreated with U0126 and PD98059 inhibitors of MEK/ERK (Fig. 8), adhesion to the 110- and 120-kDa fragments was partially blocked by both inhibitors (Figs. 8B, 8C). U0126 (1 μM) was more potent than PD98059 (50 μM) in inhibiting adhesion. U0126 blocks both active and inactive MEK/ERK, suggesting that active (U0126-inhibited but not PD98059-inhibited) MEK/ERK activity may be necessary for adhesion to the 110- and 120-kDa Fn-fs. These data suggest a role for ERK in adhesion that may be mediated solely by the cell-binding domain.

ERK Activation by Adhesion to Fn and Fn-fs

Adhesion to cellular Fn or the 70,-, 110-, and 120-kDa Fn-fs increased activation of ERK, whereas the 45-kDa Fn-f modestly increased activation when compared with untreated cells (Fig. 9A). Quantification of the p42 ERK isofrom is shown in Figure 9B. Inhibitors of PI 3-kinase were next tested for their ability to inhibit ERK activation. Both wortmannin and LY294002 reduced activation of ERK, as did the MEK inhibitor PD98059 (Fig. 9B). The 44-kDa ERK isofrom was present at lower levels in HRECs and was below the limit for linear detection by this method, which may explain the differential effects on the 44- and 42-kDa isofroms. These data indicate that ERK activation by Fn and Fn-fs occurs through a pathway that is dependent on both PI 3-kinase and MEK.

HREC Proliferation Measured by MTT Conversion

Cells plated on Fn- and Fn-f-coated dishes (0–10 μg/mL) in serum-free medium showed concentration-dependent prolifer-
ATION in response to all but the 45-kDa Fn-f. We have demonstrated that the 45-kDa Fn-f inhibits proliferation. Exposure of HRECs to the 110- and 120-kDa Fn-f resulted in a concentration-dependent increase in the number of cells (Figs. 10B, 10D, respectively). The 70-kDa Fn-f, at low concentrations, had no effect on proliferation, but at concentrations in excess of 500 ng, the 70-kDa Fn-f induced proliferation (Fig. 10A). In contrast, exposure of HRECs to c-Fn had no effect on proliferation (Fig. 10D). Similar to the adhesion process, stimulation of proliferation on different Fn-fs and c-Fn was significantly potentiated by the III1-C peptide containing a matrix assembly site (Fig. 10). The III1-C fragment alone did not have an effect on MTT conversion (data not shown).

DISCUSSION

Increased serum levels of Fn occur in diabetes, and increased amounts of Fn are detected in basement membranes of eyes in diabetes. Normalization of blood sugar levels corrects the elevated Fn and other serum abnormalities in individuals with diabetic retinopathy and delays or prevent development of the disease in individuals with no diabetic retinopathy. However, pancreatic transplantation and subsequent correction of hyperglycemia does not stabilize or reverse the retinopathy once it is established in an individual. Rather, retinopathy continues to progress in many individuals who have complete correction of diabetes after pancreatic transplantation. A possible explanation for this observation is that, although the metabolic milieu rapidly changes, endothelial cells continue to be exposed to the altered ECM that contains increased amounts of proteins such as Fn and Fn-f. In the current study, HRECs in culture expressed an increased amount of Fn when exposed to high-glucose conditions (30 mM glucose), and HRECs generated Fn-fs in vitro that modulated the activity of MMP-2 and affected proliferation and migration.

Endothelial cells responded to increased Fn by increasing expression of an integrin subtype that binds Fn (e.g., α5β1). Overexpression of the α5β1 Fn receptor reduces cell migration. Thus, increased synthesis and availability of selected integrins may mediate the proliferative effects of matrix, further confirming that integrin binding with ECM components activates intracellular pathways implicated in growth regulation. Depending on the context, integrins can transmit signals that permit or inhibit growth. In the current study, relative quantitative RT-PCR, flow cytometry and immunocytochemistry detected multiple integrins expressed by HRECs. However, there were disparities between the mRNA levels and the cell surface expression detected by flow cytometry. Although this may be a result of differential antibody affinity, the data raise the intriguing possibility that integrin levels in HRECs are subject to posttranscriptional regulation.

In the adhesion experiments, preincubation with the matrix assembly promoting III1-C peptide potentiated adhesion to all fragments, and antibodies to β1 blocked adhesion to Fn and all Fn-fs except at the highest concentration of the 70-kDa Fn-f tested. However, although the pattern of adhesion was identical in these experiments, the magnitude of adhesion observed was different between experiments (Figs. 4, 5). This suggests the possibility of donor-to-donor variation in experiments using primary cell cultures. However, results were very similar, but not identical, between donors.
FIGURE 7. Adhesion to the 70- (A), 110- (B), and 120-kDa (C) Fn-fs and c-Fn (D) was partially blocked by the PI 3-kinase inhibitors wortmannin and LY294002. Cells were pretreated for 30 minutes with 100 nM wortmannin or 50 μM LY294002 and allowed to adhere for 2 hours. Cells were then washed twice with PBS, and adhesion was quantified by MTT conversion in complete growth medium. Data represent the mean ± SE of results in four replicate experiments using four different donors (*P < 0.05; ANOVA, when compared with control data).

FIGURE 8. Adhesion to the 110- (B) and 120-kDa (C) fragments was partially blocked by the MEK inhibitors U0126 and PD98059, but adhesion to the 70kDa fragment and c-Fn was not blocked. Cells were pretreated for 30 minutes with each inhibitor and allowed to adhere for 2 hours. Nonadherent cells were washed off with two changes of PBS, and adhesion was quantified by MTT conversion in complete growth media. Data represent the mean ± SE of four replicate experiments using four different donors (*P < 0.05; ANOVA, when compared with control data).
In the current study, the β₁ integrin-blocking antibodies did not completely inhibit adhesion and were unable to block cell binding to the highest concentration of 70-kDa Fn-f tested. The 70-kDa fragment has been characterized as a matrix assembly domain. We observed this effect with two sources of antibody (Invitrogen and Chemicon) and conclude that the 70-kDa Fn fragment has been characterized as a matrix assembly domain. It is possible that this is again a result of different antibody affinities; however, it may also result from β₁-independent adhesion in the absence of functional β₁ subunits.

Fn-fs containing the RGD cell-binding domain induced proliferation that was comparable to the 120-kDa or greater than the 110-kDa Fn-f. In addition, the 70-kDa Fn-f, which does not contain the cell-binding RGD sequence, also induced HREC proliferation. Because the 45-kDa fragment is contained within the 70-kDa Fn-f and the 45-kDa Fn-f does not support proliferation or adhesion, the adhesive and mitogenic portion of the 70-kDa protein must reside within the first 30 kDa of the N terminus. This 30-kDa N-terminal fragment is generated by HRECs in culture and binds to MMP-2.

Ligation of integrins activates tyrosine kinases and small guanosine triphosphatases (GTPases) necessary for the reorganization of actin required for cell spreading. This is followed by Rho-dependent activation of cell contractility, resulting in formation of actin stress fibers, clustering of α₅β₁ integrin and assembly of mature focal adhesions.

A striking correlation exists between the actin cytoskeleton and Fn matrix organization, suggesting that the Fn matrix may be a potential modulator of actin organization functioning to influence cell signaling and growth. The assembly of focal adhesions is associated with the reorganization of Fn into fibrils. Exogenous full length Fn, as well as its 70-kDa amino terminal region of Fn, colocalize with Fn fibers. The interactions between the amino terminal region of Fn and the cell surface is the initial step in the assembly of exogenous Fn into extracellular matrix and is one of the intermolecular homophilic binding events critical for Fn polymerization. These data suggest that binding of Fn amino terminal to endothelial cells has important cytoarchitectural as well as functional consequences and that there is an intimate relationship between Fn matrix assembly and cells growth control. This Fn matrix assembly requires the activity of the integrins, α₅β₁. Fn matrix assembly also depends on self-association sites within Fn, in addition to the N-terminal 70-kDa region. The III₁-C fragment is also thought to be particularly important for the proper alignment of Fn molecules during matrix assembly.

III₁-C was also found to induce spontaneous in vitro disulfide cross-linking of Fn, to increase binding of cells to Fn and to enhance matrix assembly. Herein, we demonstrate that in HRECs, III₁-C alone had no effect but when added to Fn and Fn-fs of 110, 120, and 70 kDa, it increased proliferation, as measured by MTT conversion. Cells can adhere and spread on Fn-fs containing the RGD cell-binding domain induced proliferation or adhesion, the adhesive and mitogenic portion of the 70-kDa protein must reside within the first 30 kDa of the N terminus. This 30-kDa N-terminal fragment is generated by HRECs in culture and binds to MMP-2.

The ability of III₁-C to affect cytoskeletal function has been attributed to its ability to either disrupt preexisting Fn matrices or to stimulate increased Fn matrix deposition, depending on its concentration. The interactions between the amino terminal region of Fn and the cell surface is the initial step in the assembly of exogenous Fn into extracellular matrix and is one of the intermolecular homophilic binding events critical for Fn polymerization. These data suggest that binding of Fn amino terminal to endothelial cells has important cytoarchitectural as well as functional consequences and that there is an intimate relationship between Fn matrix assembly and cells growth control. This Fn matrix assembly requires the activity of the integrins, α₅β₁. Fn matrix assembly also depends on self-association sites within Fn, in addition to the N-terminal 70-kDa region. The III₁-C fragment is also thought to be particularly important for the proper alignment of Fn molecules during matrix assembly.

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III1-C fragment can partition to caveolin–enriched microdomains and allow signals from the ECM to be transmitted to the interior of the cell to modulate growth and contractility, and thus it is not surprising that III1-C has been shown to modulate such complex processes as cell proliferation, angiogenesis, and tumor metastasis.

The ability of each Fn-f to induce proliferation is correlated with its ability to support adhesion. Adhesion appears to be mediated by the same integrin (α5β1) for each Fn-f through a PI 3-kinase–dependent pathway. Integrin activation by Fn and Fn-fs leads to activation of the proliferation-associated kinase ERK through a pathway that is dependent on PI 3-kinase. Activation of both pathways are required for cell survival; however, although each fragment activates both pathways to a similar degree, there are differences in actin distribution in cells adherent to the fragments (Grant MB, unpublished observations, 2001). Differences between Fn-fs may exist in activation of other pathways, such as those coupled to paxillin phosphorylation that regulate cytoskeletal dynamics.

ERK activation by Fn can occur through multiple pathways. Association of ECM components with the β integrin subunit activates a signal transduction cascade, resulting in autophosphorylation of FAK on Tyr992, which creates a binding site for Src-family kinases. Src phosphorylates multiple constituents of the focal adhesion complex, including the docking protein p130Cas and FAK itself (e.g., Tyr925). Src-dependent phosphorylation of FAK at Tyr925 creates an SH2 docking site for Grb-Sos and links integrins to the ras/raf/ERK pathway in a manner that is independent of FAK. Shc phosphorylation creates an SH2 binding site for Grb-Sos and links integrins to the ras/raf/ERK pathway in a manner that is independent of FAK. Shc phosphorylation is required for integrin-stimulated proliferation and may cooperate with sustained ERK activation by FAK to cause cell cycle progression. Therefore, the Fn-fs may modulate ERK signaling through different pathways that converge upstream of ERK. For example, the 70-kDa fragment increases FAK phosphorylation (70% of the phosphorylation observed with c-Fn) but does not induce paxillin phosphorylation. This indicates either incomplete activation of FAK or differential coupling of the integrin to the transduction machinery inside the cell. Future experiments will be designed to determine the divergent pathways activated by Fn-fs that contain the cell-binding domain compared with Fn-fs that do not contain the RGD sequence.

Earlier studies have shown that cell adhesion to III1-C results in robust ERK1/2 activation and that this effect is blocked by integrin-blocking antibodies. Our observations support these findings and suggest a possible involvement of Fn assembly as a prerequisite for cellular adhesion and proliferation.

In summary, Fn and its proteolytic fragments modulate HREC adhesion and proliferation through similar signal-transduction pathways involving coupling of the α5β1 integrin through PI 3-kinase. Thus, signals from the degraded extracellular matrix provide mitogenic signals for HRECs, which may contribute to the development of diabetic retinopathy.

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