Regulation by Fibrinogen and Its Products of Intercellular Adhesion Molecule-1 Expression in Human Saphenous Vein Endothelial Cells

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Abstract—It has been reported that fibrinogen may act as a bridging ligand, binding to intercellular adhesion molecule-1 (ICAM-1) on human umbilical vein endothelial cells and to Mac-1 on THP-1 cells (a monocytic cell line) to increase adhesion. In this study, we investigated whether fibrinogen altered the expression of ICAM-1 and, thus, increased the adhesion of THP-1 cells to cultured human saphenous vein endothelial cells (HSVECs). Incubation of HSVECs with 0.3 to 4 μmol/L fibrinogen caused a time- and concentration-dependent increase in ICAM-1, as determined by ELISA. The 4- to 5-fold increase in ICAM-1 protein concentration in HSVECs stimulated by 4 μmol/L fibrinogen for 6 hours was concomitant with a 4- to 5-fold increase in ICAM-1 mRNA. This fibrinogen-stimulated ICAM-1 upregulation was associated with a 2-fold increase in THP-1 cell adhesion to HSVECs. The fibrinogen-derived peptide Bβ15-42 bound to HSVECs (Kd 0.18 μmol/L). Preincubation of HSVECs with Bβ15-42, a neutralizing antibody to urokinase plasminogen activator (uPA), or the F(ab)1 fragment of a monoclonal antibody to vascular endothelial cadherin significantly attenuated the increase in ICAM-1 stimulated by fibrinogen. Capillary electrophoretic analysis indicated that anti-uPA prevented the release of any fibrinopeptide B (Bβ1-14) in cultures of HSVECs incubated with 4 μmol/L fibrinogen for 6 hours. Moreover, incubation of HSVECs with either fibrin monomer (1 μmol/L) or monoclonal antibodies to vascular endothelial cadherin (25 μg/mL) increased ICAM-1 protein concentration 3- to 4-fold. These findings indicate that cleavage of fibrinopeptide B from fibrinogen by endothelial uPA permits the exposed Bβ15-42 to bind to vascular endothelial cadherin on HSVECs and to upregulate the expression of ICAM-1. (Arterioscler Thromb Vasc Biol. 2000;20:652-658.)

Key Words: fibrinogen ■ intercellular adhesion molecule-1 ■ saphenous vein endothelium ■ vascular endothelial cadherin ■ Bβ15–42

Fibrinogen is an important cardiovascular risk factor and contributes to elevated plasma viscosity and the high risk of fibrin clot formation in atherosclerotic vessels. An increased plasma fibrinogen concentration has been associated with saphenous vein bypass graft stenosis and occlusion.1-4 Along with the excessive proliferation of intimal smooth muscle cells, leukocyte infiltration is a major histological feature of flow-limiting stenoses in saphenous vein bypass grafts.5,6 Fibrinogen and its degradation products have several direct effects on the vascular endothelium that may be associated with the development of intimal lesions. Fibrinogen and its degradation products stimulate the release of several growth factors and urokinase plasminogen activator (uPA) from endothelial cells7,8 and can promote an increase in the vascular permeability and disorganization of the endothelium.8,9 The rapid and sustained increase in intercellular adhesion molecule-1 (ICAM-1) expression in human umbilical vein endothelial cells (HUVECs) cultured in the presence of fibrin10 could potentiate a number of cellular events mediated through this adhesion molecule. In keeping with this hypothesis, fibrinogen has been shown to mediate endothelium-dependent vasoactive effects on the saphenous vein that can be partially inhibited by a neutralizing antibody against ICAM-1.11

The demonstration that fibrinogen acts as a bridging ligand for adhesion of THP-1 cells (a monocytic cell line) to cultured HUVECs12 could signify a potential mechanism for the promotion of monocyte infiltration into the vascular wall. The role of fibrinogen as a bridging ligand was attributed to binding of the γ117-133 sequence of the fibrinogen D domain to ICAM-1 on endothelial cells.12,13 Other potential receptors on the endothelial cell surface that may bind to fibrinogen have been described; these include a receptor that recognizes an epitope within the E domain and another that recognizes the Bβ15-42 sequence near the amino terminus of fibrinogen.14,15 Very recently, it has been suggested that vascular endothelial (VE)-cadherin is the endothelial receptor that recognizes the Bβ15-42 amino terminus in the fibrin monomer.16 Arg-Gly-Asp (RGD) sequences at the carboxy termi-
nus and the middle section of the 2 Aβ chains of fibrinogen are able to bind to endothelial integrins. The functional significance of these diverse interactions of fibrinogen with the endothelium of human vessels is poorly understood. Despite the findings reported for HUVECs with subphysiological concentrations of fibrinogen (0.1 μmol/L), it is not clear whether fibrinogen has a definitive role in the promotion of monocyte adhesion in vivo. Because of our interest in the mechanisms of saphenous vein graft failure, we have investigated the possibility that incubation of cultured human saphenous vein endothelial cells (HSVECs) with high concentrations of fibrinogen is associated with the upregulation of ICAM-1. The physiological concentration of fibrinogen in whole blood is 4 to 6 μmol/L, leading to a plasma concentration of ≈10 μmol/L. In the present study, we show that prolonged incubation of HSVECs with physiological concentrations of fibrinogen (4 μmol/L) is associated with an increase in ICAM-1 expression, and we provide some evidence to indicate that this increase in ICAM-1 expression is dependent on the endothelial receptor VE-cadherin.

Methods

Primary Culture of HSVECs

The use of the saphenous vein was approved by the Riverside Research Ethics Committee. HSVECs were isolated from human saphenous vein by collagenase digestion and cultured on fibronectin-coated plastic, as described previously. HSVECs were cultured in medium 199 supplemented with 10% (vol/vol) heat-inactivated human serum, 17 U · mL⁻¹ heparin monoparin (CP Pharmaceuticals), 15 μg · mL⁻¹ endothelial cell growth supplement, 50 U · mL⁻¹ penicillin, and 50 μg · mL⁻¹ streptomycin. The human serum used was devoid of fibrinogen, as determined by immunonephelometry. Cells were transferred into serum-free medium for experiments with fibrinogen and its fragments, fibrin monomer, and antibodies, with cells from different donors being used for separate experiments.

Preparation and Assay of Fibrinogen Fragments

The complex of fragments D and E was prepared from fibrinogen (Kabi) by plasmin digestion and purified as described previously. Fragment D was prepared from the fragment D–fragment E complex by cation exchange chromatography on a Mono Q FPLC column (Pharmacia Biotech) in the presence of 10 U · mL⁻¹ Trasylol (Bayer). Fragment D was eluted by using a 0% to 70% gradient of 2 mol/L CaCl₂, 0.05 mol/L Tris-Cl, and 10 U · mL⁻¹ Trasylol. Fibrinogen fragment E was prepared from the plasmin digest after heat inactivation (30 minutes at 60°C). The supernatant collected after centrifugation at 100 000 × g for 1 hour at 10°C contained fragment E (50 kDa) and fragment E (50 kDa) were confirmed by SDS-PAGE with fragment D having been precipitated. The purity of fragment D (95 kDa) and fragment E (50 kDa) were confirmed by SDS-PAGE with use of an 8% acrylamide gel. Soluble fibrin monomer was prepared by thrombin-mediated cleavage of fibrinogen (5 μmol/L) in the presence of 2 mmol/L glycine-proline-arginine-proline as previously described. The reaction was stopped by bringing the concentration of D-phenylalaninyl-l-prolyl-l-arginyl-chloromethylketone to 1 mmol/L. Fibrinopeptides and low molecular weight reagents were removed by using Centrulplus 10 concentrators (Amicon). Fibrinopeptides A and B were assayed by capillary zone electrophoresis (Hewlett Packard) with the use of standard curves in the concentration range 0.03 to 3 μmol/L. High molecular weight fibrinogen products in cell culture medium were removed with Centrulplus 10 concentrators; the filtrate (0.5 mL) was adjusted to pH 8.0 before addition of a suspension of 100 μL Sephadex A25 (Pharmacia) to bind the fibrinopeptides. After washing of the resin with 20 mmol/L HEPES (pH 8.0), the fibrinopeptides were eluted with unbuffered 20 mmol/L HEPES, and the pH was readjusted to 7.6 before capillary electrophoresis.

ICAM-1 ELISA

This assay was adapted from the von Willebrand factor cell–based ELISA described by Short et al. In brief, cells grown in 24-well plates were fixed in methanol for 30 minutes and washed 3 times in wash buffer (10% [vol/vol] FCS and 0.5% [vol/vol] Tween 20 in PBS). Fixed cells were incubated for 40 minutes at 37°C in a 1:500 dilution of mouse anti–ICAM-1 monoclonal antibody (Dako Ltd). Cells were washed 4 times in wash buffer and incubated with 1:500 biotin-labeled secondary antibody (SeroTec) for 40 minutes at 37°C. After an additional washing, the cells were incubated with a 1:500 dilution of streptavidin horseradish peroxidase (SeroTec) for 30 minutes at 37°C before development with 0.4 mg · mL⁻¹ o-phenylenediamine (Sigma) diluted in citrate phosphate buffer (pH 5.0) containing freshly added 0.012% (vol/vol) H₂O₂. The reaction was stopped by the addition of 2.5 mol/L H₂SO₄, and the absorbance at 492 nm was measured. All assays were performed in triplicate. Cells incubated with 5 ng · mL⁻¹ interleukin (IL)-1β were used as a positive control. The absorbance resulting from background peroxidase activity and nonspecific binding (anti-caldesmon) was subtracted from all reported absorbances.

Isolation of HSVEC mRNA

HSVECs in a 75-cm² flask were incubated in the absence or presence of up to 4 μmol · L⁻¹ fibrinogen or 5 ng · mL⁻¹ IL-1β for 4 hours and washed in PBS, and mRNA was isolated by use of an mRNA microisolation kit (Sigma), which separates mRNA on oligo(dT) cellulose. The mRNA yield was 8 to 25 μg per flask.

Slot Blotting

mRNA was detected by hybridization of digoxigenin (DIG)-labeled probes. mRNA from HSVECs was diluted 1:4 to a final concentration of 50% (vol/vol) formamide and 6.7% (vol/vol) formaldehyde in SSC, incubated for 15 minutes at 68°C, and cooled on ice before application to positively charged nylon membrane in a slot-blot manifold. Wells were rinsed twice with 10 × SSC, and suction was continued for 5 minutes. Probes were labeled with DIG-dUTP with use of a DIG oligonucleotide 3′-end-labeling kit (Boehringer-Mannheim). The membrane was removed, air-dried, and baked for 2 hours at 80°C. Membranes were hybridized overnight at 54°C with either 200 ng · mL⁻¹ DIG–ICAM-1 probe cocktail (R&D Systems) or 12.5 pmol · mL⁻¹ DIG-GAPDH (Calbiochem-Novabiochem). The membrane was developed by using a DIG luminescent detection kit for nucleic acids (Boehringer-Mannheim). Quantification of mRNA was performed by densitometric scanning.

Quantification of THP-1 Cell Adhesion to HSVECs

Measurement of THP-1 cell adhesion to HSVECs was performed as described by Languno et al., except that adherent THP-1 cells were quantified by staining with a Leu M1 (CD15) monoclonal antibody (Becton-Dickinson). Briefly, passage-2 or -3 cells were grown to confluence on fibronectin-coated 24-well plates and stimulated for 6 hours with 5 ng · mL⁻¹ recombinant human IL-1β. THP-1 cells (1×10⁶ cells per milliliter) were incubated for 20 minutes at room temperature with 10 μmol/L N-formylmethionyl-leucyl-phenylalanine. In some experiments, HSVECs were preincubated with 35 μg · mL⁻¹ monoclonal antibodies against ICAM-1 or caldesmon. Medium was removed from cells, and THP-1 cells were added and incubated for 30 minutes at 37°C. Nonadhered THP-1 cells were removed, and the adhered cells were washed gently 3 times with RPMI 1640 at 37°C, followed by incubation and quantification of THP-1 cells with 100 μg · mL⁻¹ anti-CD15, as previously described.

Binding Assays

The binding of B15-42 to HSVECs was performed essentially as described previously for fibrinogen fragment D binding to HUVECs. The B15-42 peptide was labeled with [125I] by using the Iodo-Gen method (Pierce Chemical Co) to a specific activity of 20 μCi/μg. Cells, in 96-well plates, were incubated with [125I]B15-42 (0 to 750 nmol/L) in medium 199 containing 0.2% serum albumin, 17 U/mL heparin, and 15 mg/mL endothelial cell growth supplement.
at 4°C for up to 3 hours. At the end of the incubation, the medium was aspirated, and the cells were washed rapidly, 3 times, with ice-cold PBS containing 0.2% serum albumin and 1 mmol/L CaCl₂. The cells were lysed in PBS containing 1% Nonidet NP-40, frozen, and thawed once, and an aliquot (50 μL) was used for determination of radioactivity. Nonspecific binding was determined by preincubating the cells for 20 minutes with a 100-fold molar excess of Bb15-42. Specific binding was calculated as the difference in total binding and nonspecific binding.

Materials
Tissue culture medium was obtained from Life Technologies. Endothelial growth supplement, antibiotics, fibrinopeptides A and B, goat immunoglobulins, and mouse anti-caldesmon monoclonal antibody were obtained from Sigma. Fibrinectin was a gift from Dr Jan van Mourik, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB, Amsterdam, Holland). Plasminogen and streptokinase were kindly supplied by Prof David Lane, Department of Hematology, Imperial School of Medicine at Charing Cross, London, UK. Mouse anti-human tumor necrosis factor-α (TNF-α)-neutralizing antibody, recombinant human IL-1β, and ICAM-1 gene probe cocktail were obtained from R&D Systems. Mouse anti-human ICAM-1 monoclonal antibody (clone 6.5B5, IgG1 k) was from Dako, mouse anti-human VE-cadherin monoclonal antibody was from Affiniti, and polyclonal goat anti-human uPA was from Chemicon. F(ab) fragments of anti-VE-cadherin were prepared by using an Immunopure Fab Kit (Pierce & Warriner). γ peptide (corresponding to residues 117 to 113 of the fibrinogen γ chain), Bb15-42 (corresponding to residues 15 to 42 of the fibrinogen Bβ chain), Gly-Arg-Gly-Asp-Val (GRGDV), and scrambled peptides were synthesized in house, and their content was verified by mass spectrometry (Advanced Biotechnology Centre, Charing Cross Hospital). An IL-1β ELISA kit was obtained from AMS Biotechnology (Europe) Ltd.

Data Analysis
Wilcoxon signed rank tests were used for comparison of paired experiments, and ANOVA, with the Bonferroni correction, was used for multiple comparisons.

Results
Fibrinogen Increases ICAM-1 Protein Expression in HSVECs
Incubation of HSVECs with IL-1β (5 ng · mL⁻¹) for 6 hours resulted in a 5-fold increase in ICAM-1 protein concentration, as monitored by the cell-based ELISA. When HSVECs were incubated with fibrinogen (0.3 to 4 μmol/L), in the presence of 1 U · mL⁻¹ hirudin, there was a time- and concentration-dependent increase in ICAM-1 staining, although this was always less than the increase in ICAM-1 staining induced by IL-1β (Figure 1). A minimal increase in ICAM-1 staining was observed after 2 hours of incubation with IL-1β or fibrinogen, but after 6 hours of incubation with 4 μmol/L fibrinogen, ICAM-1 protein expression had increased >4-fold. This increase in ICAM-1 protein was abolished when 10 μmol/L cycloheximide or 2 μg · mL⁻¹ actinomycin was added to the culture medium.

To investigate the effect of long-term exposure of HSVECs to fibrinogen, cells were incubated with 0 to 4 μmol/L fibrinogen in serum-free medium containing 1 U · mL⁻¹ hirudin for 16 hours. Basal ICAM-1 expression (100% in the absence of fibrinogen) was increased to 154±78% at 0.3 μmol/L fibrinogen, to 171±19% at 1 μmol/L fibrinogen, to 171±28% at 2 μmol/L fibrinogen, and to 210±20% at 4 μmol/L fibrinogen (ANOVA, P<0.05; n=4). After incubation of HSVECs for >16 hours in serum-free medium, cell viability declined, with increasing concentrations of lactate dehydrogenase appearing in the medium. However, further experiments were conducted in which HSVECs were incubated with serum-free medium containing 4 μmol/L fibrinogen for 16 hours, followed by a “rest” period, during which cells were maintained in medium containing serum, but not fibrinogen, for 4 hours before further incubation with 0 to 4 μmol/L fibrinogen for a further 4 hours. Even at this time point, 1, 2, and 4 μmol/L fibrinogen caused a 2-fold increase in ICAM-1 protein expression (n=5). Therefore, the ability of fibrinogen to increase ICAM-1 in HSVECs is maintained after long-term exposure, although the increase after 16 hours was only 2-fold compared with the 4-fold increase observed on initial stimulation of HSVECs with fibrinogen.

ICAM-1 mRNA Levels Increase After Incubation of HSVECs With Fibrinogen
Incubation of cells with 5 ng · mL⁻¹ IL-1β or 0.3 to 4 μmol/L fibrinogen for 4 hours increased the ratio of ICAM-1 mRNA to GAPDH mRNA (Figure 2). After incubation of the cells with 0.3, 1, 2, and 4 μmol/L fibrinogen, there were 3.5-, 4-, 6-, and 5.7-fold increases in the ICAM-1/GAPDH mRNA ratio, respectively, compared with a 6-fold increase in the ICAM-1/GAPDH mRNA ratio after incubation of HSVECs with IL-1β.

Figure 1. Concentration and time dependence of upregulation of ICAM-1 by fibrinogen. HSVECs were incubated with fibrinogen, in the presence of hirudin (1 U · mL⁻¹), ICAM-1 protein was measured by a cell-based ELISA, and the absorbance at 492 nm is given as mean±SD of ≥6 separate experiments (using separate vein donors).

Figure 2. ICAM-1 and GAPDH mRNA in cells incubated with fibrinogen: slot blotting. Quantification of mRNA was performed by scanning densitometry. The ratio of ICAM-1 to GAPDH mRNA increases with the fibrinogen concentration, to a maximum of 5- to 6-fold at 4 μmol/L.
THP-1 Cell Adhesion Increases After Incubation of HSVECs With Fibrinogen

Very few THP-1 cells adhered to quiescent confluent monolayers of HSVECs, with the absorbance (492 nm) for CD15 detection being only 0.124±0.019. After culture of HSVECs with 4 μmol/L fibrinogen for 6 hours, the absorbance (for detection of adherent THP-1 cells) increased to 0.208±0.048 (n=5, P<0.01). Cell counting indicated that the proportion of THP-1 cells adhering to HSVECs had increased from 5 to 6% and then to 9 to 12% after incubation of HSVECs with fibrinogen. This apparent increase in THP-1 cell adhesion was abolished when HSVECs were preincubated with monoclonal antibodies against ICAM-1 (35 μg · mL⁻¹, 20 minutes) before addition of THP-1 cells (absorbance 0.146±0.037), whereas preincubation with monoclonal antibodies to caldesmon had no effect (absorbance 0.197±0.05; ANOVA, P=0.013; n=5). Although HSVECs were washed well before the addition of THP-1 cells, it was possible that traces of fibrinogen remained and served as a bridging ligand to support THP-1 cell adhesion. However, when HSVECs were preincubated with 0.3 to 4 μmol/L fibrinogen for 30 minutes before the addition of THP-1 cells, only a modest increase in THP-1 cell adhesion was observed, with maximum adhesion occurring after preincubation of cells with 0.3 μmol/L fibrinogen (ANOVA, P=0.04; n=6). THP-1 cell adhesion increased to only 118±4% of basal levels at 0.3 μmol/L fibrinogen, to 112±8% at 1 μmol/L fibrinogen, to 109±10% at 2 μmol/L fibrinogen, and to 94±4% at 4 μmol/L fibrinogen (n=6). The increase in THP-1 cell adhesion stimulated by 0.3 μmol/L fibrinogen was abolished when HSVECs were preincubated with 35 μg · mL⁻¹ anti–ICAM-1 for 30 minutes.

Fibrinogen Peptide Bβ15-42 or Antibodies to uPA Inhibit the Fibrinogen-Mediated Increase in ICAM-1 Expression

To investigate whether contamination or degradation of fibrinogen might cause the increased ICAM-1 expression in HSVECs, we conducted further experiments with ICAM-1 protein expression as the principal reporter event. The inclusion of a specific receptor, screening experiments were performed with 100 U · mL⁻¹ polymixin B (an inhibitor of endotoxin activity) in the culture medium did not attenuate the increase in ICAM-1 after incubation of HSVECs with fibrinogen. Polyacrylamide gel electrophoresis showed no major degradation of fibrinogen after 6 hours of incubation with HSVECs; the Aα chain appeared unaltered, but a small lower molecular weight satellite was observed with the Bβ chain. Fibrinopeptide A (5 μmol/L) and/or fibrinopeptide B (5 μmol/L) neither increased ICAM-1 protein expression nor inhibited the increase in ICAM-1 protein expression stimulated by fibrinogen after 6 hours of incubation. The complex of fragment D and fragment E (from plasmin degradation of fibrinogen), even at a concentration as high as 10 μmol/L, only increased ICAM-1 protein expression by <2-fold. Similarly, the individual fibrinogen fragments D and E also exhibited only weak effects on ICAM-1 protein expression. In contrast, fibrin monomer caused a concentration-dependent increase in ICAM-1 protein expression, to a maximum 3- to 4-fold increase after incubation with 1 μmol/L fibrin for 6 hours (Figure 3).

To confirm whether the effects of fibrinogen on ICAM-1 expression were secondary to the induction of IL-1β or TNF-α production by HSVECs, experiments were performed to monitor IL-1β concentrations and neutralize any TNF-α secreted. After culture of HSVECs for 6 hours in the presence of 0, 0.3, 1, 2, and 4 μmol/L fibrinogen, the concentration of IL-1β in conditioned medium was 26±7, 26±1, 23±1, 23±13, and 20±9 pg · mL⁻¹, respectively (n=3). Similarly, the ability of fibrinogen to increase ICAM-1 protein expression was maintained when HSVECs were incubated in the presence of neutralizing antibodies to TNF-α.

To investigate whether the ability of fibrinogen to increase ICAM-1 protein expression was the result of the engagement of a specific receptor, screening experiments were performed in the presence of 50 μmol/L of the following peptides: GRGDV, γ17-133 (gamma3), Bβ15-42 and scrambled Bβ15-42 (DRGAPHRPRPGPSGRSEPKEKLLPGY), and the tyrosine kinase inhibitor tyrphostin A25. None of the individual peptides increased ICAM-1 protein expression in HSVECs. Of the peptides screened, only Bβ15-42 inhibited the increase ICAM-1 protein expression stimulated by fibrinogen in HSVECs. After incubation of HSVECs with 4 μmol/L fibrinogen for 6 hours, ICAM-1 protein increased to 453±23% of the basal level. This increase in ICAM-1 protein expression was reduced to 291±18% of the basal level in the presence of 10 μmol/L Bβ15-42 ICAM-1 and to 165±20% in the presence of 50 μmol/L Bβ15-42 (ANOVA, P<0.02; n=5; Figure 3). Similarly, preincubation of HSVECs with 50 μmol/L Bβ15-42, before incubation with 1 μmol/L fibrin monomer, diminished (by >50%) the increase in ICAM-1 protein expression (Figure 3). Tyrphostin A25 (3 μmol/L) also strongly inhibited the fibrinogen-mediated increase in ICAM-1 protein expression (data not shown).
The Bβ15-42 sequence becomes exposed after enzymatic cleavage of fibrinopeptide B. Therefore, we investigated whether HSVECs expressed uPA, an enzyme known to cleave fibrinopeptide B. HSVECs showed strong positive staining for uPA (Figure 4), and preincubation of cells with a neutralizing antibody against uPA (1:5000 dilution) for 20 minutes prevented the increase in ICAM-1 protein expression stimulated by 4 μmol/L fibrinogen (Figure 3). In contrast, preincubation of cells with a similar concentration of goat immunoglobulins did not affect the 4- to 5-fold increase in ICAM-1 protein expression in response to 4 μmol/L fibrinogen (Figure 3). To determine whether cleavage of fibrinopeptide B was catalyzed by secreted uPA, HSVECs were incubated with serum-free medium for 6 hours, and this conditioned medium was incubated with fibrinogen (4 μmol/L) for 6 hours in the presence of hirudin. This fibrinogen-containing medium, in which fibrinopeptide B was not detected, was then added to HSVECs that had been preincubated with anti-uPA for 20 minutes; after 6 hours, the concentration of ICAM-1 had increased <2-fold.

Taken together, these findings indicate that cleavage of fibrinopeptide B mediated by endothelial uPA can allow the newly exposed Bβ15-42 amino-terminal domain of fibrinogen to bind to a specific receptor on HSVECs and signal for an increase in ICAM-1 protein synthesis and expression. This hypothesis is supported by the observation that fibrinopeptid B at low concentrations (0.05 to 0.08 μmol/L), but not fibrinopeptide A, was detected in the culture medium of HSVECs incubated with 4 μmol/L fibrinogen for 6 hours. Moreover, fibrinopeptide B levels fell below the limits of detection (=0.03 μmol/L) in the presence of anti-uPA (n=4). Incubation of HSVECs with a monoclonal antibody to VE-cadherin (25 μg/mL) for 6 hours also increased ICAM-1 protein expression 4- to 5-fold (n=6), whereas an isotype-matched antibody (anti-caldesmon) did not. The monoclonal antibody to VE-cadherin and fibrinogen did not have additive effects to increase ICAM-1 protein expression, but preincubation of HSVECs with Bβ15-42 (50 μmol/L) for 20 minutes before the addition of anti-VE-cadherin attenuated the increase in ICAM-1 protein expression. The absorbance at 492 nm under basal conditions and after the cells were incubated with anti-VE-cadherin and with anti-VE-cadherin + Bβ15-42 for 6 hours was 0.28±0.04, 1.19±0.18, and 0.83±0.06, respectively (n=4). In contrast, incubation of HSVECs with
the F(ab)1 fragment of anti–VE-cadherin (25 μg/mL) did not increase ICAM-1 protein expression. However, preincubation of HSVECs with this same concentration of F(ab)1 fragment for 20 minutes significantly reduced the ability of fibrinogen to increase ICAM-1 protein expression in HSVECs after 6 hours. The absorbance at 492 nm after cells were incubated with the F(ab)1 fragment alone, F(ab)1 fragment + fibrinogen (4 μmol/L), and fibrinogen alone for 6 hours was 0.25 ± 0.25, 0.58 ± 0.10, and 1.03 ± 0.12, respectively (ANOVA, P < 0.05; n = 4). These data indicate that VE-cadherin could be the fibrinogen receptor on HSVECs that signals an increase in ICAM-1 expression.

**Binding of Bβ15-42 to HSVECs**

Very high concentrations (10 to 50 μmol/L) of the Bβ15-42 peptide were required to inhibit the upregulation of ICAM-1 in HSVECs after incubation with either fibrinogen or fibrin monomer for 6 hours. The binding of [125I]Bβ15-42 to HSVECs at 4°C increased over a time period of up to 3 hours and appeared to be saturable at high concentrations of peptide (Figure 5). The Kd for Bβ15-42 binding to HSVECs at 4°C was calculated to be 0.18 μmol/L. A Scatchard plot of the ratio of bound peptide to free peptide bound [125I]Bβ15-42 was linear (Figure 5 inset), to confirm the presence of a single binding site in the concentration range studied.

**Discussion**

Providing the interface between the blood and the underlying layers of smooth muscle, the endothelium is the guardian of the vessel wall. Dejana et al. were the first to describe the specific binding of fibrinogen to the endothelium. Since then, there have been numerous reports that particular sequences on the fibrinogen molecule, or its fragments, can interact with cultured endothelial cells and result in a variety of downstream events. In particular, the cleavage of fibrinopeptide B to expose the Bβ15-42 sequence at the amino terminals of the Bβ chains has been considered to mediate the release of von Willebrand factor, endothelial cell spreading, and proliferation on fibrin. Very recently, VE-cadherin has been identified as the endothelial cell receptor for the Bβ15-42 sequence of fibrin. The present study indicates that interaction of the Bβ15-42 sequence of fibrinogen with endothelial cells cultured from human saphenous vein leads to the upregulation of ICAM-1 and increased adhesiveness of the endothelial cell monolayer.

Incubation of HSVECs with high concentrations of fibrinogen resulted in a 4- to 5-fold increase of both ICAM-1 mRNA and ICAM-1 protein within 6 hours. This upregulation of ICAM-1 had associated functional effects, namely, that the adhesion of THP-1 cells increased by 2-fold. However, fibrin monomer was also able to increase ICAM-1 protein expression in HSVECs. The magnitude, time course, and long duration of this increase in ICAM-1 expression were similar to those reported when fibrin was polymerized on HUVECs. This underscored the possibility that a degradation product of fibrinogen caused the upregulation of ICAM-1. The major fibrinogen fragments D or E or the fibrinopeptides A and B did not have comparable effects on the upregulation of ICAM-1 protein expression, and we have discounted the possibility of secondary effects mediated through upregulation of IL-1β or TNF-α production. After prolonged incubation of HSVECs with fibrinogen, very low concentrations of fibrinopeptide B (but not fibrinopeptide A) were detected. Cleavage of fibrinopeptide B exposes the 15-42 sequence at the amino terminus of the Bβ chains. The peptide corresponding to this sequence, Bβ15-42, caused a concentration-dependent inhibition of the ICAM-1 upregulation, which followed incubation of HSVECs with fibrinogen or fibrin monomer. Hence, it appeared likely that a product of fibrinogen (with fibrinopeptide B removed) at very low concentrations (~10 nmol/mL) caused the upregulation of ICAM-1 in HSVECs.

The NDSKII fibrinogen fragment, which contains the terminal Bβ15-42 sequence, binds to VE-cadherin on HUVECs (Kd 7.5 nmol/mL). Hence, nanomolar amounts of fibrinogen degradation products with the terminal Bβ15-42 sequence could bind to endothelial cells. The Bβ15-42 peptide showed much weaker binding to HSVEC monolayers (Kd 0.18 μmol/L). Nevertheless, even such weak binding could account for the partial inhibition of fibrinogen-mediated upregulation of ICAM-1 by this peptide at 10 to 50 μmol/L. The generation of fibrinogen degradation products with the terminal Bβ15-42 sequence was unlikely to be the result of thrombin activity: experiments were conducted in the presence of hirudin, and no fibrinopeptide A was detected. Another serine protease (expressed by endothelial cells) that is capable of cleaving fibrinopeptide B from fibrinogen is uPA. A neutralizing antibody to uPA prevented the upregulation of ICAM-1 and the generation of fibrinopeptide B in HSVECs incubated with fibrinogen. There are 2 pieces of evidence to indicate that the localization of the newly exposed Bβ15-42 sequence at the cell surface may be responsible for the potent effects of fibrinogen. First, only very low amounts of fibrinopeptide B (~1% fibrinogen concentration) were released over 6 hours. Second, uPA and other proteolytic enzymes secreted by HSVECs into conditioned medium did not appear to modify fibrinogen sufficiently to cause upregulation of ICAM-1 in the presence of...
neutralizing antibodies to uPA. VE-cadherin has been identified as the endothelial receptor for the Bβ15-42 sequence of fibrin. Antibodies to VE-cadherin also stimulated a 4- to 5-fold increase in ICAM-1 protein expression in HSVECs, which was partially inhibited by preincubation of the cells with the peptide Bβ15-42. More important, preincubation of HSVECs with the F(ab), fragment of anti-VE-cadherin abolished the effect of fibrinogen to increase ICAM-1 protein expression in HSVECs.

We propose that the following events are the most likely explanation for the results we have presented here: At high concentrations of fibrinogen, uPA localized on the endothelial cell surface activates the cleavage of fibrinopeptide B from fibrinogen. Subsequently, the newly exposed Bβ15-42 sequence binds to VE-cadherin on endothelial cells and stimulates intracellular events, leading to the increased expression of ICAM-1 at transcriptional and protein levels. The intracellular events are likely to include tyrosine phosphorylation signaling, because tyrosine kinase blockade inhibited the upregulation of ICAM-1 protein expression.

In summary, the present study lends further support to the hypothesis that VE-cadherin is an important endothelial receptor for fibrinogen, after cleavage of fibrinopeptide B, leading to the upregulation of ICAM-1. A similar effect of fibrinogen and its products to upregulate ICAM-1 expression on the endothelial cells remaining on a newly implanted saphenous vein graft could increase the recruitment of leukocytes and thereby influence graft patency.

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