The contact of fibrin with the apical surface of human umbilical vein endothelial cells (HUVEC) can induce capillary tube formation via the interaction of fibrin β15–42 with a putative cell receptor (Chalupowicz, D. G., Chowdhury, Z. A., Bach, T. L., Barsigian, C., and Martinez, J. (1995) J. Cell Biol. 130, 207–215). To characterize this interaction, we studied the binding of the thrombin-cleaved N-terminal disulfide knot of fibrin (NDSK II), a dimeric fragment with exposed β15–42, to HUVEC in three separate assay systems. Time-course binding of 125I-NDSK II to HUVEC monolayers or suspensions revealed that binding was specific at 50–60% as determined by the addition of unlabeled NDSK II. Specific binding of 125I-NDSK II to HUVEC was 70% reversible by dilution or by competition, and was found to be divalent cation-independent. Binding plateaued after 10 min at a saturation of 15–20 nm. Scatchard analysis using the LIGAND computer program defined a single population of receptors with a Ke of 7.7 ± 1.6 nM and approximately 21,000 ± 7000 binding sites/cell. N-terminal disulfide knot derivatives in which β15–42 was absent (NDSK 325) or unexposed (NDSK, NDSK I) did not show specific binding. Specific binding of 125I-NDSK II could not be inhibited byRGDS or by antibodies to the αvβ3 or β1 integrins, PECAM-1, ICAM-1, or N-cadherin. In contrast, a synthetic β15–42/ovalbumin conjugate inhibited total 125I-NDSK II binding by 47 ± 19% (corresponding to 95% of specific 125I-NDSK II bound) and a monoclonal antibody to vascular endothelial cadherin (VE-cadherin) inhibited binding by 35 ± 8% (corresponding to 70% of specific 125I-NDSK II bound). Another assay was based on the capture of cadherins from HUVEC lysates by a polyclonal pan-cadherin antibody immobilized on plastic dishes. Binding of NDSK II to the captured cadherins was 89 ± 5% specific, while specific binding of NDSK 325 and NDSK was negligible. An immortalized line of human adipose-derived microvascular endothelial cells, which express N-cadherin but not VE-cadherin, demonstrated no specific binding of NDSK II by the capture assay. These data define a novel interaction of fibrin with VE-cadherin, which is mediated by the fibrin N-terminal β15–42 sequence, and may contribute to the mechanism through which fibrin induces angiogenesis.

In the final steps of blood coagulation, the soluble plasma protein fibrinogen is enzymatically converted by the action of thrombin into insoluble fibrin monomers, which spontaneously polymerize to yield a fibrin mesh containing entrapped platelets and red blood cells (1). The fibrin blood clot plays a vital role in many physiologic and pathophysiologic processes including hemostasis and thrombosis (1), and in angiogenic processes such as wound healing and tumor growth (2, 3). In these processes fibrin interacts with a variety of plasma proteins, cells, and tissue matrix components. The main hemostatic function of fibrin clot formation in vivo is to provide structural support and scaffolding for the cellular and other blood elements comprising the thrombus (1). Similarly, in the process of normal wound healing, the main purpose of the fibrin clot that fills the wound is to serve as a provisional matrix into which inflammatory cells migrate and upon which new blood capillaries are formed (2). Fibrin formation is also involved in pathologic conditions, such as tumor growth, where it is thought to provide a matrix for tumor cell proliferation and possibly for maintenance of angiogenesis (2).

The interaction of the N-terminal region of fibrin with the endothelial cell surface influences cell behavior in several respects. It can disrupt the normal integrity of the endothelial cell monolayer (4), it can stimulate secretion of stored von Willebrand factor (5) and it can induce human umbilical vein endothelial cell (HUVEC) morphogenesis into capillary tube-like structures (6, 7), thus initiating the process of in vitro angiogenesis. These observations indicate that the thrombin-mediated exposure of neo-epitopes at the N terminus of the fibrin molecule confers fibrin, as opposed to fibrinogen, with the ability to bind to endothelial cell surface receptors and thereby induce the specific cellular responses referred to above.

We have previously shown that a specific form of fibrin designated fibrin II, in which the neo-N terminus of the fibrin β chain (β15–42) is exposed, induces the formation of capillary tube-like structures when in contact with the apical surface of HUVEC monolayers (7). We have also more recently demonstrated that a monoclonal antibody against the extracellular domain of VE-cadherin, or cadherin-5 (8), inhibited fibrin II and collagen-induced capillary tube formation whereas antibodies against N-cadherin, PECAM-1, or the αvβ3 integrin did not (9). In related experiments, others have shown that antibodies against VE-cadherin or PECAM-1 do not inhibit capillary tube formation when used individually, but do inhibit tube formation when incubated together with endothelial cells cul-

* This work was supported in part by National Institutes of Health Grant HL-20092 (to J. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Dunglison M.D./Ph.D. candidate; recipient of a predoctoral fellowship from the American Heart Association, Southeastern Pennsylvania Affiliate.

‡ To whom correspondence should be addressed: Cardeza Foundation for Hematologic Research, Thomas Jefferson University, 1015 Walnut St., Philadelphia, PA 19107. Tel.: 215-955-8458; Fax: 215-923-3836; E-mail: jos.martinez@mail.tju.edu.

1 The abbreviations used are: HUVEC, human umbilical vein endothelial cells; VE-cadherin, vascular endothelial cadherin; PECAM-1, platelet endothelial cell adhesion molecule-1; Ig, immunoglobulin family of cell adhesion molecules; ICAM-1, intercellular cell adhesion molecule-1; HADMEC, human adipose-derived microvascular endothelial cells; NDSK, N-terminal disulfide knot of fibrinogen; TBS, Tris-buffered saline.
VE-cadherin Is a Receptor for Fibrin

In the present investigation, we focused our attention on the interaction of the N terminus of fibrin, namely the β15–42 sequence, with an endothelial cell receptor by characterizing the binding of the N-terminal disulfide knot (NDSK) of fibrin to HUVEC. We have also utilized a cadherin capture assay to analyze the direct interaction of fibrin with isolated cadherins. Based on our results, we propose that VE-cadherin serves as a fibrin-specific receptor on endothelial cells and that the N terminus of the fibrin β chain (β15–42) functions as a heterophilic ligand for VE-cadherin.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—Medium 199 with Earle’s salts and Hepes buffer, Dulbecco modified Eagle’s medium, and collagenase type I were purchased from Life Technologies, Inc. RPMI 1640 medium was purchased from Fisher Scientific. Carrier-free 125I (NaI in NaOH) and the ECL detection system were purchased from Amersham Pharmacia Biotech. Silicone oil (DC 550) was purchased from Dow Corning Corp. Human fibrinogen was purified as described previously (26). To prepare the NDSK, fibrinogen (10 mg/ml) was dialyzed into 70% formic acid and was treated with cyanogen bromide (1.3 mg/100 mg of fibrinogen) for 24 h at room temperature. The solution was dialyzed into 50 mM Tris-buffer, pH 7.4, and contaminating hydrophobic fragments (28) were removed by centrifugation at 10,000 × g. The supernatant, containing two major cleavage products, was chromatographed on a Sephadex G-100 column, and fractions eluted after the void volume were pooled and analyzed by SDS-PAGE. Purified NDSK was treated with thrombin (3 units/ml for 3 h at 37 °C) to generate NDSK II (NDSK lacking fibrinopeptides A and B). NDSK I (NDSK minus fibrinopeptide A) was prepared by treating NDSK with Atraxin (2 μg/ml for 2 h at 37 °C) and NDSK 325, which lacks β15–42, was prepared by treatment of NDSK with protease III (8 μg/ml for 3 h at 37 °C) from C. atrax venom (29). The identities of the NDSK derivatives were assessed by SDS-PAGE. For binding experiments, NDSK fragments were labeled with iodine 125, by the IODIGEN method (30), to a specific activity of 3000–5000 cpm/μg.

**Synthesis of the β15–42 Peptide Sequence**—The β15–42 sequence, corresponding to the neo-N terminus of the fibrin β chain following cleavage of fibrinopeptide B by thrombin, was synthesized by the Fmoc (N-(9-fluorenyl)methoxycarbonyl) strategy of solid-phase synthesis (31) using an Applied Biosystems 430A peptide synthesizer and was purified by reversed-phase chromatography using an Applied Biosystems semipreparative system equipped with a Rainin C18 column. The sequence of the purified β15–42 peptide was verified by mass spectroscopy. The purified peptide was conjugated to ovalbumin by glutaraldehyde cross-linking (12).

**Binding Assays**—Binding of NDSK derivatives to HUVEC suspensions was performed essentially as described previously for fibrinogen binding to HUVEC (26). Briefly, HUVEC (∼250,000 cells/ml) suspended in buffer containing 1% bovine serum albumin and no added cations, were incubated in 12-mm multil wells with the 125I-NDSKs at a final concentration of 20 nM. The suspensions were incubated with horizontal shaking at 4 °C and aliquots of suspension were removed in duplicate and added to microcentrifuge tubes containing a mixture of 1 part light mineral oil and 4 parts DC550 silicone oil (26). The cells were pelleted by centrifugation at 12,000 × g for 10 min, the tips of the tubes were amputated, and the cell-bound radioactivity was counted by γ-scintillation spectrometry. Specific binding was determined by subtracting nonspecific binding from total binding. Specific binding was determined for 20 min with a 100-fold molar excess of unlabeled NDSK, NDSK I, or NDSK 325, prior to the addition of the respective 125I-labeled derivatives.

Specific binding was calculated from the difference in total binding and nonspecific binding. Reversibility of binding was assessed either by dilution with excess binding buffer or by displacement with excess...
unlabeled NDSK II. To assess the cation dependence of the system, binding studies were carried out with binding containing added CaCl₂ (2 mM), MgCl₂ (2 mM), MnCl₂ (0.5 mM), or EDTA (8 mM). To assess the integrity of the cell-bound 125I-NDSK, the radioactive pellet was extracted into Laemmli sample buffer (32), electrophoresed, and subjected to autoradiography as described below.

Potential inhibitory agents, such as antibodies (5–10 µg/ml) to endothelial cell integrins, cadherins, or Ig superfamily molecules, or specific fibrin-derived peptides such as µ15–42 (25 µg/ml) or RGDS (0.5 mM), were preincubated with the cells for 20 min prior to the addition of the 125I-NDSKs. For assessment of the potential inhibitory role of heparin, heparin was preincubated at increasing concentrations with the fibrinopeptide A-modified 125I-NDSK II (33). Following incubation of HUVEC with the 125I-NDSK fragments, binding was quantitated as described above.

For the studies in monolayer, HUVEC were grown to confluence on 5-cm wells (~ 500,000 cells/well), washed twice with 2 ml of serum-free Medium 199 containing 1% bovine serum albumin, pH 7.4, and preincubated with the same medium for 15 min at 37 °C prior to the addition of the NDSK derivatives. All monolayer studies were carried out at 37 °C. Nonspecific binding was determined as described above for cell suspensions. Following incubation, duplicate wells were washed twice with TBS and detached with TBS/EDTA, 900-µl aliquots of detached cells were added to microcentrifuge tubes containing a mineral oil/DCC550 silicone oil mixture, and the cell-bound 125I-NDSK was quantitated as described above for the suspension studies.

Estimation of Binding Parameters—Scatchard plots of NDSK II binding to HUVEC were analyzed by curve-fitting software developed by Munson and Rodbard (34) for estimation of dissociation constants (Kₐ) and maximal binding (Bmax). The data were analyzed initially using both a two-site and a one-site model with nonspecific binding set at zero. Convergence of the data was obtained with only the one-site model. The data were subsequently analyzed by a one-site model with nonspecific binding set as a parameter proportional to the ligand concentration. The experimental estimates of nonspecific binding, determined from the binding of labeled ligand in the presence of 100-fold molar excess of unlabeled ligand, coincided within 15% of estimates by curve fitting using the one-site model.

Co-immunoprecipitation of VE-cadherin and NDSK II—Immunoprecipitation was performed as described previously (35) with some modifications. HUVEC monolayers (10–15 T-75 flasks; ~30–45 × 10⁶ cells) were washed three times with TBS containing 2 mM CaCl₂ and then scraped, on ice, into TBS, pH 7.4, containing 2 mM CaCl₂, 1 mM phenylmethylsulfonyl fluoride, 15 µg/ml aprotinin, and 15 µg/ml leupeptin (lysis buffer). The samples were centrifuged for 5–10 min at 1000 × g and the supernatants were resuspended in 0.5 ml of the lysis buffer containing 0.5% SDS and 1% Nonidet P-40, put on ice for 15 min, and aspirated back and forth several times through a 27.5-gauge needle. After 15 min, lysis buffer containing only 1% Nonidet P-40 was added to bring the final concentration of SDS to 0.1%, the samples were allowed to stand on ice for 15 min and then centrifuged at 12,000 × g for 15 min at 4 °C. To the supernatant, 125I-NDSK II was added to a final concentration of 30 nM and the mixtures were capped and rotated end-over-end for 20–30 min at room temperature. The NDSK II-bound lysate was then incubated for 2 h on ice with either an anti-pan-cadherin IgG or a control rabbit IgG, both at a final concentration of 50 µg/ml. Subsequently, 100 µl of the Protein A-Sepharose suspension was added to each sample and incubated for 60 min on an end-over-end shaker. The incubation mixture was centrifuged for 1–2 min at 12,000 × g and the pellet washed four times with 1 ml of lysis buffer. After the final wash, the pellets were boiled in Laemmli sample buffer (32), electrophoresed, and immunoblotted as described above.

Results

Analysis of NDSK Fragments—Fibrinogen can be cleaved with cyanogen bromide to generate a fragment termed the NDSK, which is composed of the intact N termini of the Aα, Bβ, and γ chains held together by disulfide bonds. The NDSK can be treated with thrombin, Atroxin, or protease III to generate modified NDSK derivatives, (i.e. NDSK II, NDSK I, or NDSK 325, respectively). Table I summarizes the composition of these derivatives. Each NDSK fragment, when run under nonreducing conditions on 12% SDS-PAGE, appears as a heterogeneous group of bands as depicted (Fig. 1A). The heavier, lightestaining bands have been observed in other studies involving CNBr degradation of fibrinogen (28, 37), and are most likely due to the presence of oxidized methionine residues that are resistant to CNBr cleavage thus resulting in incomplete cleavage at the C termini of the fragments (37). The N-terminal residues, however, remain completely intact as demonstrated by N-terminal sequence analysis of fibrinogen NDSK, fibrin NDSK I, and fibrin NDSK II (37, 38). Thus, as the N-terminal residues of fibrinogen-derived NDSK are cleaved with either thrombin (Fig. 1, lane 2), Atroxin (Fig. 1, lane 9), or protease III (Fig. 1, lane 4), the entire group of bands migrates at their new lower molecular weights compared with the untreated fibrinogen NDSK (Fig. 1, lane 1). The major bands of NDSK, NDSK II, NDSK I, and thrombin-treated NDSK 325 migrate at 63.7, 58, 61, and 54 kDa, respectively (Fig. 1A).

The same NDSK fragments were transferred to nitrocellulose after running on SDS-PAGE and Western blots were performed using a monoclonal antibody (T2G1) to the β15–21 sequence in order to identify the antigenic exposure of this sequence (36). As shown in Fig. 1B, although present in NDSK (lane 1), NDSK II (lane 2), and NDSK I (lane 3), the β15–21 sequence is available as an antigenic epitope only in NDSK II (lane 2), which was generated by cleavage of fibrinopeptide B with thrombin.

Table I

| Starting material | Cleaving enzyme | Peptides released | Resulting material |
|------------------|----------------|------------------|-------------------|
| NDSK             | Thrombin       | FPA + FPB        | NDSK II           |
| NDSK             | Atroxin        | FPA              | NDSK I            |
| NDSK             | Protease III   | Bβ 1–42          | NDSK 325          |
| NDSK II          | Protease III   | FPA + Bβ 1–42    | Thrombin-cleaved  |
| NDSK II          | Thrombin       | FPA + FPB        | NDSK 325          |

VE-cadherin Is a Receptor for Fibrinogen

Fibrinogen can be cleaved with cyanogen bromide to generate a fragment termed the NDSK, which is composed of the intact N termini of the Aα, Bβ, and γ chains held together by disulfide bonds. The NDSK can be treated with thrombin, Atroxin, or protease III to generate modified NDSK derivatives, (i.e. NDSK II, NDSK I, or NDSK 325, respectively). Table I summarizes the composition of these derivatives. Each NDSK fragment, when run under nonreducing conditions on 12% SDS-PAGE, appears as a heterogeneous group of bands as depicted (Fig. 1A). The heavier, lightestaining bands have been observed in other studies involving CNBr degradation of fibrinogen (28, 37), and are most likely due to the presence of oxidized methionine residues that are resistant to CNBr cleavage thus resulting in incomplete cleavage at the C termini of the fragments (37). The N-terminal residues, however, remain completely intact as demonstrated by N-terminal sequence analysis of fibrinogen NDSK, fibrin NDSK I, and fibrin NDSK II (37, 38). Thus, as the N-terminal residues of fibrinogen-derived NDSK are cleaved with either thrombin (Fig. 1, lane 2), Atroxin (Fig. 1, lane 9), or protease III (Fig. 1, lane 4), the entire group of bands migrates at their new lower molecular weights compared with the untreated fibrinogen NDSK (Fig. 1, lane 1). The major bands of NDSK, NDSK II, NDSK I, and thrombin-treated NDSK 325 migrate at 63.7, 58, 61, and 54 kDa, respectively (Fig. 1A).

The same NDSK fragments were transferred to nitrocellulose after running on SDS-PAGE and Western blots were performed using a monoclonal antibody (T2G1) to the β15–21 sequence in order to identify the antigenic exposure of this sequence (36). As shown in Fig. 1B, although present in NDSK (lane 1), NDSK II (lane 2), and NDSK I (lane 3), the β15–21 sequence is available as an antigenic epitope only in NDSK II (lane 2), which was generated by cleavage of fibrinopeptide B with thrombin.
Characterization of NDSK Binding to HUVEC—Because fibrin II, but not fibrin I or fibrin 325, can induce endothelial cell capillary tube formation (7) which can be inhibited by antibodies to VE-cadherin (9), we hypothesized that fibrin II may bind specifically to endothelial cell receptors, possibly VE-cadherin. However, because our preliminary binding studies were complicated by the problem of fibrin polymerization, 2 we chose to study the binding of various NDSKs to HUVEC, eliminating the binding of fibrin polymers since the NDSKs are soluble. Furthermore, since the purified NDSKs do not contain the RGD sequences present in the native molecule, potential RGD-dependent interactions are likely eliminated. This approach also afforded us the opportunity to characterize the binding determinants expressed at the N terminus of fibrin and to aid in the identification of an endothelial cell fibrin receptor.

Time-course binding studies conducted over 3 h revealed that the total binding of 125I-NDSK II (20 nM) to HUVEC suspensions at 4 °C or monolayers at 37 °C reached a plateau level in 10 min (Fig. 2). Therefore, in subsequent experiments, incubations were carried out for 20 min prior to determination of particular binding parameters. Fig. 2 also demonstrates that total binding in monolayer saturates at a lower number of molecules per cell than in suspension (5,000 versus 40,000, respectively).

Specific binding of 125I-NDSK II by dilution was observed to be reversible, reaching a maximum of 70% displacement after 2.5 h (Fig. 3). Reversibility by competition, using excess unlabeled NDSK II, gave identical results (data not shown). Moreover, the 125I-NDSK II recovered from bound cells ran predominantly as a 58-kDa band, unchanged from the appearance of the starting material (data not shown) indicating that the radioactivity bound to HUVEC in these studies was NDSK II which did not undergo significant degradation or polymerization.

The relative affinities of the 125I-labeled and unlabeled NDSK II were compared by conducting isotope dilution experiments in which the concentration of total NDSK II was kept constant at 25 nM while the proportion of unlabeled NDSK II to 125I-NDSK II was progressively increased. This type of analysis revealed that the affinities of the two ligands were very similar, as evidenced by a linear decrease in bound 125I-NDSK II as its proportion decreased (data not shown).

2 T. L. Bach, C. Barsigian, C. H. Yaen, and J. Martinez, unpublished observations.

Saturability of NDSK Binding to HUVEC—Binding isotherms of 125I-NDSK II from 2.5 to 25 nM to HUVEC suspensions were performed and analyzed by the LIGAND computer program (34). The data defined a single population of receptors that saturated near 20 nM (Fig. 4), and Scatchard data of five separate experiments revealed an apparent dissociation constant (Kd) of 7.7 ± 1.6 nM with approximately 21,000 ± 7000 binding sites/cell (Fig. 4, inset).

The specific binding of 125I-NDSK II to HUVEC monolayers reached a saturation between 10 and 20 nM (data not shown), similar to that of the suspension studies (Fig. 4). However, the extent of binding was much less than in suspension and the accuracy of binding at subsaturating concentrations was limited by the low number of counts bound. Therefore, binding specificity was determined at saturating concentrations as described below.
Specificity of NDSK II Binding to HUVEC—To establish the specificity of NDSK binding to HUVEC, the binding of each radiolabeled NDSK fragment (20 nM) was measured in the absence or presence of a 100-fold molar excess of its corresponding unlabeled fragment. As demonstrated in Fig. 5A, only the binding of the thrombin-cleaved \(^{125}\text{I}-\text{NDSK II}\) was significantly inhibited by its cold fragment, revealing specific binding of 51 ± 6%. Similar results were obtained with confluent HUVEC monolayers at 37 °C as shown in Fig. 5B, which demonstrates that the specific binding of NDSK II was 69 ± 5%, but that the number of molecules bound per cell was 10-fold less than in suspension, as previously noted for the time-course experiments (Fig. 2). The decreased number of NDSK II molecules in monolayer compared with suspension is possibly attributable to a decrease in the number of VE-cadherin molecules available for heterophilic binding as a result of strong homophilic binding to other VE-cadherin molecules present at intercellular contact sites.

Since neither NDSK nor NDSK I (which contain β15–42 masked either by fibrinopeptides A and B or fibrinopeptide A alone, respectively), or NDSK 325 (which lacks β15–42) manifest specific binding, these results suggest that the association of the N terminus of fibrin II with the endothelial cell is most likely mediated by the β15–42 sequence, which becomes functionally unmasked only upon cleavage of fibrinopeptide B (see Fig. 1B). To assess this possibility more directly, we conducted binding studies with excess β15–42 peptide as an inhibitor. When the β15–42 sequence was cross-linked to ovalbumin and preincubated with HUVEC suspensions, binding of \(^{125}\text{I}-\text{NDSK II}\) was inhibited to the level observed with 100-fold molar excess of unlabeled NDSK II, whereas inhibition with ovalbumin alone was insignificant (Fig. 6). These data support the concept that β15–42 mediates specific binding of NDSK II to HUVEC.

Divalent Cation Influence on NDSK II Binding to HUVEC—Calcium and manganese have been reported to influence fibrinogen binding to β₁ and β₃ integrins (16, 39); therefore, we considered whether these cations might also influence the binding of NDSK II to HUVEC. In these experiments, \(^{125}\text{I}-\text{NDSK II}\) (20 nM) was incubated with HUVEC suspensions with added CaCl₂, MgCl₂, MnCl₂, or EDTA, and total binding was measured after 20 min of incubation at 4 °C. The addition of divalent cations or EDTA failed to either stimulate or inhibit \(^{125}\text{I}-\text{NDSK II}\) binding (data not shown), establishing that NDSK II binding to HUVEC is a divalent cation-independent process.

NDSK II Binds to VE-cadherin on HUVEC—In order to elucidate the receptor to which NDSK II binds, several agents were tested for their ability to inhibit the binding of \(^{125}\text{I}-\text{NDSK II}\) to HUVEC. These included antibodies to the various cell adhesion molecules known to be present on endothelial cells, specific peptides present within fibrin(ogen), and heparin, which has recently been shown to inhibit binding of fibrin fragments to HUVEC (33). The results of these experiments revealed that binding of \(^{125}\text{I}-\text{NDSK II}\) to HUVEC could not be inhibited by the antibodies 7E3 or LM 609 (data not shown), both of which are directed against the integrin α₁β₃ (15, 40). In agreement with these findings, the RGDS peptide, which corresponds to the integrin-binding domain of fibrinogen, also failed to inhibit NDSK II binding to HUVEC (data not shown). Taken together, these data support the conclusion that integrins did not mediate NDSK II binding to HUVEC under the conditions tested. Unlike previous studies of fibrin N-terminal fragment binding to HUVEC (33), heparin (5–500 µg/ml) did not inhibit the binding of NDSK II to HUVEC in our system, suggesting that the fibrin receptor is not a heparan proteoglycan and that binding of heparin to the heparin-binding domain (β15–42) did not interfere with NDSK II binding to the fibrin receptor of endothelial cells.

As depicted in Fig. 7, of the antibodies tested, only a monoclonal antibody directed against the first two extracellular domains of VE-cadherin was capable of inhibiting \(^{125}\text{I}-\text{NDSK II}\) binding to HUVEC. In contrast, antibodies against the extracellular domain of N-cadherin did not significantly inhibit NDSK II binding (Fig. 7). Similarly, the binding of NDSK II to HUVEC was not influenced by monoclonal antibodies against PECAM-1 or ICAM-1 (data not shown), indicating that these two members of the Ig superfamily do not serve as receptors for the N terminus of fibrin II.

Because the VE-cadherin antibody inhibited NDSK II binding, we further analyzed the role of VE-cadherin in fibrin binding by studying the association of NDSK II with cells that do not express VE-cadherin. For these experiments, cultured vascular human smooth muscle cells were lysed, run in SDS-PAGE, and immunoblotted using the VE-cadherin antibody. These experiments showed that VE-cadherin was not expressed by these cells (data not shown), as previously shown by other investigators (9, 22). More importantly, these venous smooth muscle cells did not show specific binding of \(^{125}\text{I}-\text{NDSK II}\) whether in suspension at 4 °C or as monolayers at 37 °C (data not shown).

**VE-cadherin Is a Receptor for Fibrin**
Fig. 5. Specific binding of various 125I-NDSK derivatives to HUVEC.
Specific binding of each 125I-NDSK derivative was determined by incubation for 20 min at 4 °C with HUVEC suspensions (A) or 37 °C with HUVEC monolayers (B), with (Nonspecific) or without (Total Bound) a prior 20-min incubation with a 100-fold molar excess of each respective unlabeled NDSK fragment. 125I-NDSKs were added to a final concentration of 20 nM. Binding of 125I-NDSKs was determined following centrifugation of cells through silicone oil. 125I-labeled NDSK II in the absence of inhibitor (CONT), unlabeled NDSK II (100X), NDSK, protease III-treated; NDSK I, Atroxin-treated; NDSK 325, protease III-treated. Values for total and nonspecific binding represent the means ± S.D. of duplicate measurements from four experiments. *, p < 0.05.

VE-cadherin Is a Receptor for Fibrin

To further establish the role of VE-cadherin as the NDSK II receptor of endothelial cells, we used a polyclonal pan-cadherin antibody to assess whether 125I-NDSK II would co-immunoprecipitate from HUVEC lysates along with VE-cadherin. The pan-cadherin antibody was used since it is directed at the conserved cytoplasmic domain of the cadherins and should therefore not interfere with the extracellular NDSK II binding site, as would be expected for the monoclonal antibody which inhibited NDSK II binding (Fig. 7) and which is directed against the extracellular domain of VE-cadherin. The Western blots of the immunoprecipitated 130-kDa HUVEC cadherin are demonstrated in Fig. 8 (panels A and B), using the polyclonal pan-cadherin and monoclonal VE-cadherin antibodies, respectively. The large bands at the bottom of lanes 2 and 3 in panel A migrate at a molecular mass of 50 kDa and likely represent the IgG of the immune complexes after being run under reducing conditions. Panel C demonstrates that the pan-cadherin antibody immunoprecipitated 59 ± 18% more radioactive NDSK II than did a nonimmune IgG. Therefore, just as in the binding studies (Fig. 5), the amount of specific binding of 125I-NDSK II to isolated endothelial cell cadherins is approximately 50–60%.

Isolated HUVEC Cadherins Specifically Bind NDSK II—To

Fig. 6. Inhibition of 125I-NDSK II binding to HUVEC by a β15–42-ovalbumin conjugate. HUVEC suspensions were preincubated with either unlabeled NDSK II (2 μM), the β15–42 peptide coupled to ovalbumin (25 μg/ml) or ovalbumin alone (25 μg/ml) for 20 min at 4 °C. 125I-NDSK II was added to a final concentration of 20 nM and allowed to proceed for 20 min at 4 °C. Binding of 125I-NDSK II was then determined following centrifugation of cells through silicone oil. 125I-labeled NDSK II in the absence of inhibitor (CONT), unlabeled NDSK II (100X), β15–42 coupled to ovalbumin (β15–42-OVA), and ovalbumin (OVA). Binding was expressed as counts per minute (CPM) of 125I-NDSK II bound to HUVEC. The data shown are means ± S.D. of duplicate measurements from four experiments. *, p < 0.05.

Fig. 7. Effect of anti-cadherin monoclonal antibodies on 125I-NDSK II binding to HUVEC. HUVEC suspensions were preincubated with unlabeled NDSK II (2 μM), or monoclonal antibodies (5–10 μg/ml) against N-cadherin (clone NCAD2) or VE-cadherin (clone 75) for 20 min prior to addition of 125I-NDSK II (20 nM) and further incubation for 20 min at 4 °C. Binding of 125I-NDSK II was determined following centrifugation of cells through silicone oil. 125I-labeled NDSK II in the absence of antibody (CONT), unlabeled NDSK II (100X), anti-VE-cadherin (VE-CAD), anti-N-cadherin (N-CAD). Binding was expressed as counts per minute (CPM) of 125I-NDSK II bound to HUVEC. The data shown are means ± S.D. of duplicate measurements from four experiments. *p < 0.05.

Further establish the identity of VE-cadherin as the NDSK II receptor of HUVEC, we used a modified antigen capture assay in which HUVEC cadherins were immobilized in wells coated with the polyclonal pan-cadherin antibody. The Western blots of the immunocaptured 130-kDa HUVEC cadherin is demonstrated in Fig. 9A using the monoclonal VE-cadherin antibody. The results shown in Fig. 9B illustrate that the binding of 125I-NDSK II to captured cadherins reached a plateau at 20 nM and that specific binding, as determined by preincubation of wells with a 100-fold molar excess of unlabeled NDSK II, was 89 ± 5% (Fig. 9C). Fig. 9C also shows that the binding of 125I-NDSK and 125I-NDSK 325 to captured cadherins was negligible, and that the binding of 125I-NDSK II by material captured by nonimmune IgG was only 11 ± 8%, similar to the level of nonspecific binding of NDSK II. Thrombin-treated 125I-NDSK 325 also failed to bind to captured cadherins (data not shown), indicating that exposure of the neo-N terminus of the α chain was not involved in binding to VE-cadherin. The higher level of specific binding of 125I-NDSK II in this system, as compared with the binding suspension experiments and immunoprecipitations, is probably due to the capacity of the immunocapture method to reduce nonspecific binding by removal of other cell surface molecules that are washed away after immunocapture.
The conclusion that the $^{125}$I-NDSK II was in fact bound to captured endothelial cell VE-cadherin is supported by the observation that Western blot analysis of material scraped from the capture assay plate coated with the pancadherin polyclonal antibody revealed VE-cadherin that was not detected from the plate coated with control rabbit IgG (Fig. 9A, lanes 2 and 3, respectively). Although it is also likely that N-cadherin was captured using the polyclonal pan-cadherin antibody, captured N-cadherin could not be demonstrated by Western blots possibly due to the low sensitivity of the N-cadherin antibody as revealed by Western blot analysis with HUVEC lysates (data not shown). As a final demonstration of the role of VE-cadherin in binding of fibrin to endothelial cells is difficult to quantitate since fibrin forms insoluble polymers after release of fibrinopeptides from fibrinogen (1). In order to circumvent the binding of polymerized fibrin, the soluble NDSK of fibrin, which lacks the polymerization sites at the C terminus of the fibrin molecule, was used to explore the structural requirements of fibrin binding to endothelial cells in vitro. NDSK is a fragment of fibrinogen, generated by cleavage with cyanogen bromide, which retains the N-terminal structural features of each of the fibrin chains consisting of the dimeric fragment of A0 1–51, Bβ 1–118, and γ 1–78 (27, 38). Various forms of the NDSK lacking either fibrinopeptide A and B (NDSK II), fibrinopeptide A only (NDSK I), or the sequence Bβ1–42 (NDSK 325) can be prepared by treatment of the NDSK of fibrinogen with either thrombin, Atroxin, or protease III, respectively.

The results of our investigation clearly demonstrate that NDSK II, which contains the neo-N-terminal A15–42 sequence, specifically binds with high affinity to HUVEC suspensions in

**DISCUSSION**

The present paper focuses on the binding of soluble fibrin derivatives to HUVEC. The analysis of binding of fibrin itself to
sequence at the C terminus of fibrinogen (18, 19). In our present study, however, we found that antibodies against PECAM-1 or ICAM-1 did not inhibit the binding of NDSK II to endothelial cells, in agreement with our previous report of the failure of anti-PECAM-1 antibodies to inhibit fibrin II-induced capillary tube formation (9). Of note, the $\gamma_{117–133}$ sequence that binds to ICAM-1 is lost upon cleavage of fibrinogen with cyanogen bromide, thus eliminating the possibility of its interference in the binding of NDSK II to endothelial cells.

It has been recently demonstrated that CNBr-cleaved N-terminal fragments of fibrin bind to HUVEC and that preincubation of the N-terminal fragments with heparin inhibits the binding (33). These findings suggest that the heparin binding domain, previously identified as $\beta_{15–42}$ (49), is likely involved in mediating the observed binding of fibrin fragments, potentially to a heparan sulfate-containing proteoglycan (33). In contrast, in our system, preincubation of purified NDSK II with heparin did not have any appreciable effect on the binding of NDSK II. These differences may be due to differences in the experimental systems. Alternatively, it may be that the binding of NDSK II to HUVEC VE-cadherin has a higher affinity than does the putative proteoglycan-like receptor, and so is preferentially active in our studies.

Cadherins are adhesive molecules present at adherens junctions, which mediate cell-cell contacts via their extracellular domains while anchoring to cytoskeletal structures via their cytoplasmic tails (24, 50–52). They play a major role in morphogenesis (50, 53, 54) and development of blood vessels during embryogenesis (25). Vascular endothelial cells express two different cadherins (20, 22), N-cadherin and VE-cadherin. While N-cadherin appears to be diffusely distributed over the plasma membrane (20), VE-cadherin is known to be located at intercellular junctions of essentially all types of endothelium (23, 24) and has recently been shown to mediate endothelial cell capillary tube formation in vitro either on its own (9) or in conjunction with PECAM-1 (10).

We have recently demonstrated that a monoclonal antibody against VE-cadherin, but not N-cadherin, prevents formation of endothelial cell capillary tubes induced by sandwiching HUVEC between fibrin-fibrin or collagen-collagen gels (9). Moreover, the VE-cadherin monoclonal antibody also produced disruption of preformed capillary tubes. These data indicate that fibrin may function as a heterophilic ligand for VE-cadherin on
VE-cadherin Is a Receptor for Fibrin

The mechanisms involved in the interaction of fibrin with VE-cadherin, which results in the formation of capillaries, have not been fully elucidated. It is possible that the N-terminal region of fibrin associates with apically expressed VE-cadherin molecules, which are not present in intercellular junctions, and that this association induces intracellular signals directly associated with endothelial cell morphogenesis. It is also possible that the initial disruption of endothelial cell monolayers, which occurs with overlying polymerized fibrin (4), permits the interaction of fibrin with VE-cadherin molecules present in the intercellular junctions, and that it is these topographically restricted associations which are responsible for the assembly of endothelial cells into capillary tubes.

In this investigation we have identified endothelial cell VE-cadherin as a receptor for the β15–42 sequence at the neo-N terminus of the fibrin β chain exposed upon thrombin-mediated cleavage of fibrinopeptide B. Thus, we have identified both a new fibrin-specific receptor as well as the first heterophilic ligand for VE-cadherin that demonstrates binding independent of divalent cations. Because of the central role of fibrin in blood coagulation, wound healing, and tumor growth and of VE-cadherin in endothelial cell function, these findings have broad implications in the understanding of the fibrin association with vascular endothelium in processes such as hemostasis and thrombosis, as well as in signaling cascades which may play a role in a variety of endothelial cell functions including the induction of capillary-tube formation during the angiogenic process.

Acknowledgments—We thank Andrew S. Likens for the artwork, Rosemarie Silvano for preparation of the manuscript, and Bonny Lightner and Heather Ryan for their experience in cell culture.

REFERENCES

1. Moesson, M. W. (1992) Semin. Hematol. 29, 177–188
2. Dvorak, H. F. (1986) N. Engl. J. Med. 315, 1650–1658
3. Arnold, F., and West, D. C. (1991) Pharmacol. Ther. 52, 407–422
4. Weinier, B., and Delvos, U. (1986) Arteriosclerosis 6, 139–145
5. Rübs, J. A., Ni, F., Wagner, D., and Franzis, C. W. (1989) J. Clin. Invest. 84, 435–442
6. Dvorak, H. F., Harvey, S., Estrella, P., Brown, L. F., McDonagh, J., and Dvorak, A. M. (1987) Lab. Invest. 57, 673–683
7. Chalupowicz, D. G., Chowdhury, Z. A., Bach, T. L., Barsigian, C., and Martinez, J. (1995) J. Cell Biol. 120, 207–215
8. Suzuki, S., Sano, R., and Tanahara, H. (1991) Cell Regul. 2, 261–270
9. Bach, T. L., Barsigian, C., Chalupowicz, D. G., Bowler, D., Yasen, C. H., Grant, D. S., and Martinez, J. (1998) Exp. Cell Res. 238, 324–334
10. Matsumura, T., Wolfe, K., and Petzelbauer, P. (1997) J. Immunol. 158, 3408–3416
11. DeLasser, H. M., Christophidou-Solomidou, M., Strietier, R. M., Burdick, M. D., Robinson, C. S., Wexler, R. S., Kerr, J. S., Garlanda, C., Merwin, J. R., Madri, J. A., and Albeida, S. M. (1997) Am. J. Pathol. 151, 671–677
12. Erban, J. K., and Wagner, D. (1992) J. Biol. Chem. 267, 696–698
13. Bischoff, J. (1995) Trends Cell Biol. 5, 69–74
14. Stromblad, S., and Chen, D. A. (1996) Trends Cell Biol. 6, 462–467
15. Chen, D. A., Berliner, S. A., Vezene, V., and Ruggeri, Z. M. (1989) Cell 58, 945–953
16. Suehiro, K., Galili, J., and Plow, E. F. (1997) J. Biol. Chem. 272, 5360–5366
17. Newman, P. J. (1997) J. Clin. Invest. 100, 525–529
18. Languino, L. R., Plescia, J., Duperray, A., Brian, A. A., Plow, E. G., Galletsky, J. E., and Altieri, D. C. (1993) Cell 73, 1423–1434
19. Altiери, D. C., Duperray, A., Plescia, J., Thornton, G. B., and Languino, L. R. (1990) J. Biol. Chem. 265, 8042–8048
20. Salomon, D., Avalon, O., Patel-King, R., Hynes, R. O., and Geiger, B. (1992) J. Cell Sci. 102, 7–17
21. Lampugnani, M. G., Resnati, M., Baiter, M., Pioget, R., Pascaone, A., Housen, G., Roco, L. P., and Dejana, E. (1992) J. Cell Biol. 118, 1511–1522
22. Breviario, F., Caveda, L., Corada, M., Martini-Padura, I., Navarro, P., Gelay, J., Introna, M., Golin, D., Lampugnani, M. G., and Dejana, E. (1995) Arterioscler. Thromb. Vasc. Biol. 15, 1229–1239
23. Dejana, E., Corada, M., and Lampugnani, M. G. (1995) FASEB J. 9, 910–918
24. Dejana, E. (1997) J. Clin. Invest. 100, S7-S10
25. Vittet, D., Buchou, T., Schütz, A., Dejana, E., and Huber, P. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 6273–6278
26. Martinez, J., Rich, E., and Barsigian, C. (1989) J. Biol. Chem. 264, 20502–20508
27. Bloombak, B., Blomback, M., Hessel, B., and Iwanaga, S. (1967) Nature 215, 1445–1448
28. Shainoff, J. R., Stearns, D. J., DiBello, P. M., and Hishikawa-Itoh, Y. (1990) J. Cell Biol. 111, 1603–1609
29. Pandy, B. V., and Budzynski, A. Z. (1984) Biochemistry 23, 460–470
30. Fraker, P. M., and Speck J. C. (1978) Biochem. Biophys. Res. Commun. 80, 849–857
VE-cadherin Is a Receptor for Fibrin

31. Chang, C. D., and Meienhofer, J. (1978) *Int. J. Pept. Protein Res.* **11**, 246–249
32. Laemmli, U. K. (1970) *Nature* **227**, 680–685
33. Odrjini, T. M., Francis, C. W., Sporn, L. A., Bunce, L. A., Marder, V. J., Simpson-Haidaris, P. J. (1996) *Arterioscler. Thromb. Vasc. Biol.* **16**, 1544–1551
34. Munson, P. J., and Rodbard, D. (1986) *Ann. Biochem.* **107**, 220–239
35. Lampugnani, M. G., Corada, M., Caveda, L., Breviario, F., Ayala, O., Geiger, B., and de Jeana, E. (1995) *J. Cell Biol.* **129**, 203–217
36. Procyk, R., and Kudryk, B. (1991) *Blood* **77**, 1469–1475
37. Meh, D. A., Siebenlist, K. R., Bergstrom, G., and Mosesson, M. W. (1995) *Lab. Clin. Med.* **125**, 384–391
38. Blomback, B., Blomback, M., Henschen, A., Hessel, B., Iwanga, S., and Woods, K. R. (1986) *Nature* **218**, 933–943
39. Smith, J. W., Piotrowicz, R. S., and Mathis, D. (1994) *J. Biol. Chem.* **269**, 960–967
40. Oster, Z. H., Srivastava, S. C., Som, P., Meinken, G. E., Seubler, L. E., Yamamoto, K., Atkins, H. L., Drill, A. B., and Celler, B. S. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 3435–3438
41. Flynn, J. T., Westbrooks, M., and Lucas, K. A. (1997) *Shock* **7**, 1–10
42. Madri, J. A., and Pratt, B. M. (1986) *J. Histochem. Cytochem.* **34**, 5–91
43. Grant, D. S., Tashiro, K-I, Segui-Real, B., Yamada, Y., Martin, G. R., and Kleinman, H. K. (1989) *Cell* **58**, 933–943
44. Brooks, P. C., Clark, R. A. F., and Cheresh, D. A. (1994) *Science* **264**, 569–571
45. Friedlander, M., Brooks, P. C., Shaffer, R. W., Kincaid, C. M., Varner, J. A., and Cheresh, D. A. (1995) *Science* **270**, 1500–1502
46. Vernon, R. B., and Sage, R. H. (1995) *Am. J. Pathol.* **147**, 873–883
47. Ruoslahtti, E., and Pierschbacher, M. D. (1986) *Cell* **44**, 517–518
48. Langurin, L. R., Colella, S., Zanetti, A., Andreux, A., Rykewaert, J. J., Charon, M. H., Marchiolo, P. C., Plov, E. F., Ginsberg, M. H., Marguerie, G., and de Jeana, E. (1989) *Blood* **73**, 734–742
49. Odrjini, T. M., Shainoff, J. R., Laurence, S. O., Simpson-Haidaris, P. J. (1996) *Blood* **88**, 2050–2061
50. Geiger, B., and Ayalon, O. (1992) *Annu. Rev. Cell Biol.* **8**, 307–332
51. Suzuki, S. T. (1996) *Cell Biol. Biochem.* **61**, 531–542
52. Takeichi, M. (1991) *Science* **251**, 1451–1455
53. Gumbiner, B. M. (1996) *Cell* **84**, 345–357
54. Mengaud, J., Ohayon, H., Gounon, P., Mege, R.-M., and Cossart, P. (1996) *Cell* **84**, 923–932
55. Karela, P. I., Green, S. J., Bowden, S. J., Coadwell, J., and Kilshaw, P. J. (1996) *J. Biol. Chem.* **271**, 30999–30915
56. Famiglietti, J., Sun, J., DeLisser, H. M., and Alfelda, S. M. (1997) *J. Cell Biol.* **138**, 1425–1435
57. Brieher, W. M., Yap, A. S., Gumbiner, B. M. (1996) *J. Cell Biol.* **135**, 487–496