BIOCHEMISTRY
including biophysical chemistry & molecular biology
Article

Biochemistry **2009,** *48,* 5171–5179 5171
DOI: 10.1021/bi900487d

Interaction of Fibrin(ogen) with the Endothelial Cell Receptor VE-Cadherin: Localization of the Fibrin-Binding Site within the Third Extracellular VE-Cadherin Domain†

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Received March 20, 2009; Revised Manuscript Received May 4, 2009

ABSTRACT: Interaction of fibrin with endothelial cells through their receptor VE-cadherin has been implicated in modulation of angiogenesis and inflammation. Previous studies identified the VE-cadherin-binding site in the fibrin βN-domains formed by the NH_2-terminal regions of fibrin β chains and revealed that the recombinant dimeric $(\beta 15-66)_2$ fragment mimicking these domains preserves the VE-cadherin-binding properties of fibrin. To test if the other fibrin(ogen) regions/domains are involved in this interaction and localize the complementary fibrin-binding site in VE-cadherin, we prepared several recombinant fragments containing individual extracellular domains of VE-cadherin or combinations thereof, as well as several fragments corresponding to various fibrin(ogen) regions, and tested the interactions between them by ELISA and surface plasmon resonance. The experiments revealed that the βN-domains are the only fibrin(ogen) regions involved in the interaction with VE-cadherin. They also localized the fibrin-binding site to the third extracellular domain of VE-cadherin and established that the fibrin-binding properties of this domain are not influenced by the presence or absence of the neighboring domains. In addition, the experiments confirmed that calcium ions, which are required to maintain proper conformation and adhesive properties of VE-cadherin, do not influence the fibrin-binding properties of the latter.

Plasma protein fibrinogen, the major component of the blood clotting system, is converted to fibrin by thrombin, a blood clotting enzyme generated upon activation of the blood coagulation cascade in response to vascular injuries or other stimuli. Fibrin polymerizes spontaneously to form insoluble clots that seal the injured vasculature, thereby preventing the loss of blood. In addition to its hemostatic function, fibrin also plays a prominent role in wound healing by promoting physiological inflammation and angiogenesis through its interactions with leukocytes and endothelial cells (*1*−*6*). Because of its proinflammatory and proangiogenic properties, fibrin may also contribute to pathological inflammation and tumorigenesis (*7*−*9*).

Interaction of fibrin(ogen) with leukocytes and endothelial cells is mediated by numerous cell receptors. It was suggested that fibrin(ogen), by interacting with the leukocyte receptor Mac-1 ($\alpha M\beta 2$ integrin) and endothelial cell receptor ICAM-1, bridges inflammatory cells to the endothelium, thus promoting their transendothelial migration and thereby inflammation (*1*, *10*). Fibrin may also bridge leukocytes to the endothelium through the interaction with the endothelial cell receptor VE-cadherin[1] (*11*). Further, it was demonstrated that fibrin promotes the formation of capillary tubes by the endothelial cell monolayer and this process involves fibrin−VE-cadherin interaction (*3*, *12*, *13*). Thus, the interaction of fibrin with endothelial cells through VE-cadherin has been implicated in modulation of both fibrin-dependent inflammation and angiogenesis. The mechanisms by which such interaction modulates these processes are not established yet.

Fibrinogen is a complex multidomain protein consisting of two identical subunits, each of which is formed by three nonidentical polypeptide chains, Aα, Bβ, and γ (*14*) (Figure 1A). The NH_2-terminal portions of all six chains are linked together in the central region of the molecule by 11 disulfide bonds forming the so-called N-terminal disulfide knot, NDSK, which is preserved in the NDSK fragment prepared by CNBr digestion of fibrinogen (*14*, *15*). Upon conversion of fibrinogen into fibrin, thrombin removes two short peptides, fibrinopeptide A and fibrinopeptide B, from the NH_2 termini of the Aα and Bβ chains, respectively. The removal of fibrinopeptide B (Bβ chain residues 1−14) is required for the exposure of the VE-cadherin-binding site (*12*). This site was originally localized in the 15−42 portion of the fibrin β chain (*12*, *13*). However, a synthetic β15−42 peptide corresponding to this portion inhibited fibrin-induced angiogenesis only at very high concentrations (*12*),

†This work was supported by National Institutes of Health Grant HL-56051 to L.M.

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[1]Abbreviations: VE-cadherin, vascular endothelial cadherin; HRP, horseradish peroxidase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; SPR, surface plasmon resonance; TBS, 20 mM Tris buffer, pH 7.4, containing 150 mM NaCl; HBS-P, 10 mM HEPES buffer, pH 7.4, containing 150 mM NaCl and 0.005% surfactant P20; Ab, antibodies; mAb, monoclonal antibody.

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and its affinity to endothelial cells was lower than that of the thrombin-treated NDSK fragment, NDSK II (*3*, *13*, *16*). Because this peptide represents roughly half of the fibrin βN-domain, which includes residues β15−57 (*17*), we prepared a recombinant fragment, β15−64, corresponding to the full-length βN-domain, and a dimeric $(\beta15-66)_2$ fragment, which mimics the dimeric arrangement of this domain in fibrin, and studied their binding to VE-cadherin (*18*). The study revealed that two βN-domains are required for a high-affinity interaction with VE-cadherin to occur and that their His16 and Agr17 are critical for binding (*18*). Whether other fibrin(ogen) domains are involved in the interaction with VE-cadherin remains to be tested.

Vascular endothelial cadherin (VE-cadherin, cadherin-5) is a member of the cadherin superfamily of adhesion transmembrane receptors that have homologous structures and are involved in Ca^{2+}-dependent, homophilic interactions[2] (*19*, *20*). VE-cadherin belongs to the type II cadherins, which are highly related to the classical type I cadherins and share with them similar domain structure (*21*, *22*). The NH_2-terminal extracellular portion of VE-cadherin contains five homologous repeats of about 110 residues each, which form five extracellular domains, followed by transmembrane and cytoplasmic domains (*23*, *24*) (Figure 1B). VE-cadherin mediates cell−cell contacts at intercellular junctions via homophilic interactions between its extracellular domains, while its cytoplasmic domain interacts with several intracellular proteins that couple VE-cadherin to the actin cytoskeleton (*19*, *20*). Besides the homophilic interactions, the extracellular portion of VE-cadherin also interacts with fibrin (*3*, *13*). A monoclonal antibody, which recognizes an epitope within the first two extracellular domains of VE-cadherin, was capable of inhibiting NDSK II binding to endothelial cells, suggesting the involvement of these domains in fibrin binding (*13*). In contrast, our study revealed that only a recombinant VE-cadherin fragment corresponding to the first four extracellular domains interacts with fibrin and its $(\beta15-66)_2$ fragment, while that corresponding to the first two domains exhibited no fibrin-binding activity (*18*). Thus, localization of the fibrin-binding site in VE-cadherin is still unclear.

The major goals of this study were to further characterize the interaction between fibrin and VE-cadherin, establish which of the extracellular domains of VE-cadherin are involved in this interaction, and clarify whether the βN-domains account for all the VE-cadherin binding capacity of fibrin or other fibrin regions/domains are also involved.

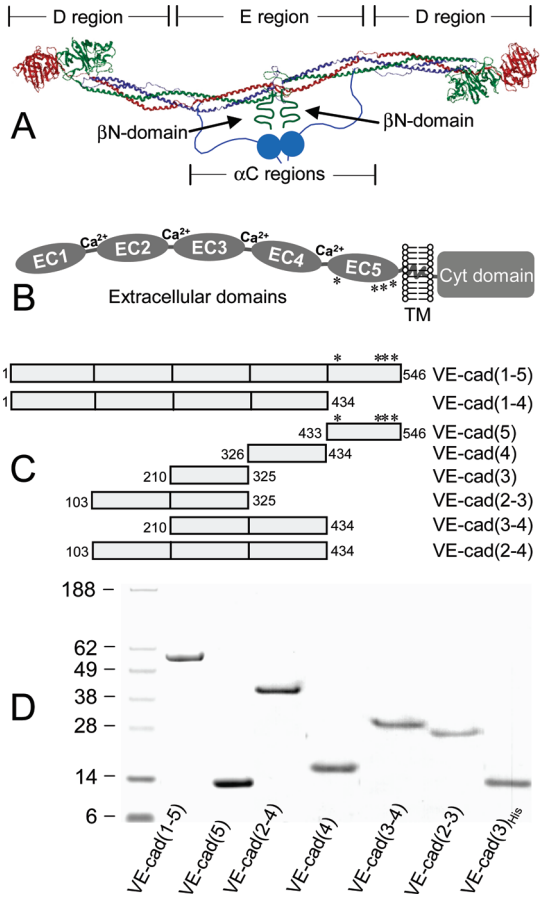

FIGURE 1: Schematic representation of fibrinogen, VE-cadherin, and recombinant VE-cadherin fragments prepared for this study. Panel A: Ribbon diagram of fibrinogen based on its crystal structure (*55*); the individual fibrinogen chains, Aα, Bβ, and γ, are colored blue, green, and red, respectively. The αC regions and βN-domains, whose structures have not been identified, are shown schematically as two blue spheres attached to the bulk of the molecule with flexible connectors and two curved green lines, respectively. The vertical lines denote approximate boundaries between the D, E, and αC regions of fibrinogen. Panel B: A diagram of VE-cadherin consisting of five extracellular domains, transmembrane domain (TM), and cytoplasmic domain (Cyt); four Cys residues forming two disulfide bonds in the fifth extracellular domain are shown by asterisks; calcium ions bound at the short linkers connecting the extracellular domains are also depicted. Panels C and D: Recombinant VE-cadherin fragments and their SDS−polyacrylamide gel electrophoresis analysis, respectively; the left outer lane in panel C contains protein markers of the indicated molecular masses.

EXPERIMENTAL PROCEDURES

Proteins, Enzymes, and Antibodies. Human fibrinogen (plasminogen, vWF, and fibronectin depleted), plasmin, and α-thrombin were from Enzyme Research Laboratories (South Bend, IN). The T2G1 monoclonal antibody against the fibrin β15−21 region (*25*, *26*) was a gift from Dr. B. Kudryk (New York Blood Center). The antihuman VE-cadherin goat polyclonal antibodies (anti-hVE-cadherin AF938 Ab) were from R&D Systems (Minneapolis, MN). The horseradish peroxidase-(HRP-) conjugated donkey anti-goat polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). HisProbe-HRP, a nickel-activated derivative of HRP, was from Pierce.

Preparation of Recombinant and Proteolytic Fibrin(ogen) Fragments. The recombinant $(B\beta1-66)_2$ fragment was produced in *Escherichia coli* and purified as described earlier (*18*). To produce the $(\beta15-66)_2$ fragment, we used a procedure described in ref (*18*) with some modifications. Briefly, $(B\beta1-66)_2$ was treated with a 50% suspension of thrombin−agarose from the Trombin CleanCleave Kit (Sigma) for 2 h at room temperature and subsequently purified by gel filtration on Superdex-75. The recombinant αC fragment (residues Aα221−610) corresponding to the fibrinogen αC region was produced in *E. coli* and subsequently purified and refolded as described in ref (*27*). The fibrinogen-derived E_3 fragment and fibrin-derived D-D and E_1 fragments were prepared from plasmin digests of fibrinogen and fibrin, respectively, by the procedures described in refs (*28*) and (*29*).

Preparation of Recombinant VE-Cadherin Fragments. Recombinant VE-cadherin fragments, VE-cad(1−5), VE-cad(1−5)$_{His}$, VE-cad(1−4), VE-cad(2−4), VE-cad(2−3), VE-cad(3−4),

[2]Homophilic interaction is an interaction between two identical components.

VE-cad(3), VE-cad(3)$_{His}$, VE-cad(4), and VE-cad(5), including amino acid residues 1−546, 1−546 tagged with six His residues (His tag), 1−434, 103−434, 103−325, 210−434, 210−325, 210−325 with His tag, 326−434, and 433−546, respectively, were produced in *E. coli* strain BL21(DE3)pLysS using the pET-20b expression vector (Novagen). The cDNA fragments encoding these regions were produced by PCR using as a template the full-length cDNA encoding human VE-cadherin, which was kindly provided by Dr. J. Martinez (*18*), and the primers are summarized in Table S1 of Supporting Information. The PCR products were subcloned into the pET20b expression vector using *Nde*I and *Xho*I restriction sites and then transformed into DH5α *E. coli* host cells (Invitrogen). It should be noted that the presence of stop codon in reverse primers 2, 4, and 6 (Table S1) prevented translation of the plasmid's His-tag coding sequence. All resulting clones were sequenced to confirm the integrity of the coding sequences. For production of the recombinant fragments, the BL21/pLysS *E. coli* host cells were transformed with the resulting plasmids, and all fragments were produced and refolded following the procedures described earlier (*30*). The purity of the fragments was verified by SDS−polyacrylamide gel electrophoresis (SDS−PAGE). The VE-cad(3) and VE-cad(4) fragments were additionally sequenced and analyzed by mass spectroscopy (see Results). The NH_2-terminal sequence analysis was performed using the Procise 494 HT protein sequencing system (Applied Biosystems); all MALDI-TOF mass spectra were acquired on an Axima-CFR plus instrument (Shimadzu).

Protein Concentration Determination. Concentrations of the newly expressed VE-cadherin fragments were determined spectrophotometrically using theoretical extinction coefficients ($E_{280,1\%}$) estimated from fragment sequences by the ProtParam online tool (http://www.expasy.ch/tools/protparam.html). Molecular masses of these fragments were also estimated using ProtParam. The following molecular masses and $E_{280,1\%}$ values were obtained: VE-cad(1−5), 61.8 kDa and 8.4; VE-cad(1−5)$_{His}$, 62.5 kDa and 8.3; VE-cad(1−4), 49.3 kDa and 9.3; VE-cad(2−4), 37.5 kDa and 7.4; VE-cad(2−3), 25 kDa and 6; VE-cad(3−4), 26 kDa and 8.1; VE-cad(3), 13.4 kDa and 6.7; VE-cad(3)$_{His}$, 14.1 kDa and 7.4; VE-cad(4), 12.7 kDa and 9.5; VE-cad(5), 12.9 kDa and 2.5. Concentrations of the previously described fragments, VE-cad(1−2) and (β15−66)$_2$, were determined as in ref (*18*).

Solid-Phase Binding Assay. Wells of Immulon 2HB microtiter plates were coated overnight at 4 °C with the (β15−66)$_2$ fragment at 2 μg/mL in 0.1 M Na_2CO_3, pH 9.5 (coating buffer). The wells were then blocked with SuperBlock buffer (Pierce) for 1 h at room temperature. Following washing with TBS (20 mM Tris, pH 7.4, 150 mM NaCl) containing 0.05% Tween 20 and 1 mM $CaCl_2$ (ELISA-binding buffer), the indicated concentrations of the VE-cadherin fragments in this buffer were added to the wells and also to control wells coated with just SuperBlock and incubated overnight at 4 °C. Bound fragments were detected by reactions with specific goat anti-hVE-cadherin polyclonal antibodies AF938 (45 min at 37 °C) and the HRP-conjugated donkey anti-goat polyclonal antibodies (45 min at room temperature). The peroxidase substrate, SureBlue TMB (KPL, Gaithersburg, MD), was added to the wells, and the amount of bound ligand was measured spectrophotometrically at 450 nm. Data were analyzed by nonlinear regression analysis using the equation:

$$A = A_{max}/(1 + K_d/[L]) \quad (1)$$

where A represents the absorbance of the oxidized substrate, which is assumed to be proportional to the amount of ligand bound, A_{max} is the absorbance at saturation, [L] is the molar concentration of the ligand, and K_d is the dissociation constant. It should be noted that in the control experiments AF938 Ab recognized all VE-cadherin fragments prepared for this study.

The inhibition effect of the (β15−66)$_2$ fragment on the binding of VE-cadherin to fibrin was studied using the VE-cad(1−5)$_{His}$ fragment containing His tag. Wells of Immulon 2HB microtiter plates were coated overnight at 4 °C with fibrinogen at 5 μg/mL in the coating buffer and then incubated with 0.1 NIH/mL thrombin for 1 h at 37 °C to convert fibrinogen into fibrin. Following washing with the binding buffer, the wells were blocked with SuperBlock for 1 h at room temperature. VE-cad(1−5)$_{His}$ at 100 nM in the binding buffer was preincubated with increasing concentrations of (β15−66)$_2$ for 1 h at 37 °C, and 100 μL aliquots of the mixtures were added to the wells and incubated at 4 °C overnight. Bound VE-cad(1−5)$_{His}$ was detected by the reaction with HisProbe-HRP, as recommended by the manufacturer.

To test the specificity of interaction between the VE-cad(3)$_{His}$ and (β15−66)$_2$ fragments, wells of Immulon 2HB microtiter plates were coated overnight at 4 °C with (β15−66)$_2$ at 2 μg/mL in the coating buffer. The wells were blocked as above, VE-cad(3)$_{His}$ at 200 nM in the binding buffer was preincubated with an equimolar amount of T2-G1 mAb for 1 h at 37 °C, and 100 μL aliquots of the mixture were added to the wells and incubated overnight at 4 °C. Bound VE-cad(3)$_{His}$ was detected by the reaction with HisProbe-HRP.

Surface Plasmon Resonance Analysis. Interaction of the fibrin(ogen) fragments with the VE-cadherin fragments was studied by surface plasmon resonance (SPR) using the BIAcore 3000 biosensor (Biacore AB, Uppsala, Sweden), which measures the association/dissociation of proteins in real time. Immobilization of the VE-cadherin fragments to the activated surface of the CM5 sensor chip was performed using the amine coupling kit (BIAcore AB), as specified by the manufacturer. Binding experiments were performed in HBS-P (10 mM HEPES buffer, pH 7.4, containing 150 mM NaCl and 0.005% surfactant P20) with 1 mM $CaCl_2$ or 2 mM EDTA at 10 μL/min flow rate. The fibrin (ogen) fragments were injected at increasing concentrations, and the association/dissociation between them and the immobilized VE-cadherin fragments was monitored as the change in the SPR response. To regenerate the chip surface, complete dissociation of the bound fibrin(ogen) fragments was performed as described earlier (*18*). Experimental data were analyzed using BIAevaluation 4.1 software supplied with the instrument. The dissociation equilibrium constant, K_d, was calculated as $K_d = k_{dissoc}/k_{assoc}$, where k_{assoc} and k_{dissoc} represent kinetic constants that were estimated by global analysis of the association/dissociation data using the 1:1 Langmurian interaction model (kinetic analysis). To confirm the kinetic analysis, K_d was also estimated by analysis of the association data using the steady-state affinity model provided by the same software (equilibrium analysis).

RESULTS

Expression and Characterization of VE-Cadherin Fragments. Our previous study revealed that the VE-cad(1−4) fragment including the first four extracellular domains of VE-cadherin interacts with fibrin and the (β15−66)$_2$ fragment mimicking fibrin βN-domains, while the VE-cad(1−2) fragment containing the first two domains does not interact (*18*). This

finding suggests that the third and/or fourth domains are important for the interaction; however, it does not exclude that the other domains, especially the fifth one that has never been tested for fibrin binding, may also be involved. To test this speculation and further localize the fibrin-binding site, we expressed a VE-cad(1−5) fragment including all five extracellular domains of VE-cadherin and a number of fragments containing its individual domains or combinations thereof (Figure 1C).

While analyzing the newly expressed fragments by SDS−PAGE, we noticed that electrophoretic mobility of the VE-cad (4) fragment was slower than that of VE-cad(3), in spite of its lower expected molecular mass (not shown). Additional analysis of these fragments by mass spectroscopy revealed that while VE-cad(4) had the expected molecular mass (observed, 12709; expected, 12703), the observed molecular mass of VE-cad(3) was about 1 kDa lower than the expected one (observed, 12356; expected, 13284). Because the NH_2-terminal sequence analysis of VE-cad(3) revealed the presence of the intact NH_2 terminus, we concluded that the COOH-terminal portion of this fragment corresponding to the residues Asp318−Phe325 is missing. This truncation most probably occurred during the expression and purification of this fragment. At the same time, the addition of His tag to the COOH terminus of VE-cad(3) prevented this truncation. Namely, when we constructed and expressed a VE-cad$(3)_{His}$ fragment including such a tag, this fragment preserved its ability to react with the His probe and therefore contained the complete sequence of the third domain.

The SDS−PAGE analysis of all fragments used in this study, which demonstrates their purity, is presented in Figure 1D. Note that electrophoretic mobility of VE-cad(4) was still slower than that of the slightly larger VE-cad$(3)_{His}$ fragment. Since all VE-cadherin fragments were expressed in bacteria and denaturing concentrations of urea were used upon their purification and refolding, it was important to test their folding status. When the fragments were heated in a fluorometer or spectropolarimeter while monitoring respectively the ratio of fluorescence intensities or ellipticity at a fixed wavelength, as previously described (*18*, *31*), they all exhibited sigmoidal transitions reflecting unfolding (not shown). Thus all fragments were folded into a compact structure.

Interaction of the VE-Cad(1−5) Fragment with Fibrin(ogen) Fragments. Previous studies revealed the involvement of the fibrin βN-domains in the interaction with VE-cadherin (*13*, *18*). To test whether other fibrin(ogen) regions/domains are involved, we studied the interaction of the VE-cad(1−5) fragment with the fibrin-derived D-D and E_1 fragments, fibrinogen-derived E_3 fragment, and the recombinant Aα221−610 fragment. Note that the D-D, E_1, and Aα221−610 fragments correspond to the fibrin(ogen) D, E, and αC regions, respectively, and thus, together they represent essentially the complete fibrin(ogen) molecule (Figure 1A). In surface plasmon resonance (SPR) experiments, when these fragments were added to VE-cad(1−5) covalently immobilized onto the surface of a sensor chip, only E_1 exhibited a prominent binding while D-D and Aα221−610 failed to bind; the E_3 fragment, which differs from E_1 mainly by the absence of the βN-domains, also displayed no binding (Figure 2A). These results indicate that VE-cadherin interacts with fibrin only through its βN-domains.

To confirm this finding, we studied binding of VE-cad(1−5) to immobilized fibrin in the presence of increasing concentrations of the $(\beta15-66)_2$ fragment mimicking the βN-domains by ELISA. In these competition experiments, we first used specific

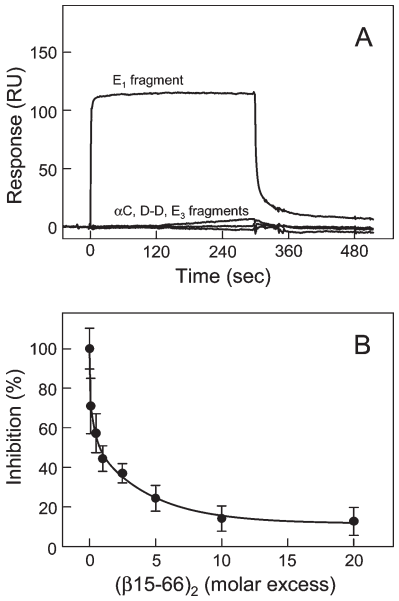

FIGURE 2: Identification of fibrin(ogen) regions that interact with VE-cadherin. Panel A: Binding of fibrin(ogen) fragments to the immobilized VE-cad(1−5) fragment detected by surface plasmon resonance. The fibrin(ogen)-derived D-D, E_1, and E_3 fragments, or the recombinant Aα221−610 fragment, all at 1 μM in HBS-P buffer with 1 mM $CaCl_2$ (see Experimental Procedures) were added to immobilized VE-cad(1−5), and their association/dissociation was monitored in real time by registering the resonance signal (response). Panel B: Inhibition effect of the $(\beta15-66)_2$ fragment on the binding of the VE-cad$(1-5)_{His}$ fragment to immobilized fibrin detected by ELISA. VE-cad$(1-5)_{His}$ in ELISA-binding buffer was incubated with increasing concentrations of $(\beta15-66)_2$, and bound VE-cad $(1-5)_{His}$ was detected with the His-tag probe, as described in Experimental Procedures. Data are expressed as a percentage of control binding in the absence of competing $(\beta15-66)_2$ and are means ± the standard deviation of triplicate determinations.

anti-hVE-cadherin goat Ab and secondary HPR-conjugated donkey anti-goat Ab for detection of bound VE-cad(1−5). However, in such detection system some interaction of these antibodies with immobilized fibrin was observed. To overcome this problem, we constructed and expressed a VE-cad(1−5) fragment with His tag, VE-cad$(1-5)_{His}$, which enabled direct detection of the bound fragment with the His-tag probe (see Experimental Procedures). Using this detection system, we found that $(\beta15-66)_2$ inhibited binding of VE-cad$(1-5)_{His}$ to fibrin in dose-dependent manner and at a 20-fold molar excess almost completely abrogated this binding (Figure 2B). In another ELISA experiment, in which we used the same detection system, VE-cad$(1-5)_{His}$ added at 2.5 μM to the immobilized D-D, E_3, αC, or $(\beta15-66)_2$ fragments exhibited binding only to $(\beta15-66)_2$ (data not shown). Altogether, these results reinforce the above finding that the βN-domains are the only regions of fibrin interacting with VE-cadherin.

Testing of Fibrin-Binding Properties of the Fifth Extracellular Domain of VE-Cadherin. To test the involvement of the fifth domain of VE-cadherin in the interaction with fibrin, we studied binding of the VE-cad(5) fragment corresponding to this domain to the $(\beta15-66)_2$ fragment. In ELISA, wells of microtiter plates were coated with $(\beta15-66)_2$, incubated with increasing concentrations of the VE-cad(5) or VE-cad(1−5) fragments, the latter was used as a control, and the bound fragments were detected with anti-hVE-cadherin Ab. No binding of VE-cad(5) to $(\beta15-66)_2$ was observed even when the former was added at 500 nM while VE-cad(1−5) exhibited dose-dependent binding

with K_d = 44 nM (Figure 3 and Table 1). These results indicate that the isolated fifth domain of VE-cadherin does not interact with fibrin.

It should be noted that the value of K_d = 44 nM for the interaction of $(\beta15-66)_2$ with VE-cad(1−5) determined in this study was about 3-fold lower than the K_d value of 120 nM determined earlier by ELISA for the interaction of $(\beta15-66)_2$ with the VE-cad(1−4)$_{His}$ fragment (*18*). This suggests that the fifth domain, which does not interact with fibrin being isolated, may still contribute to the fibrin-binding properties of the extracellular portion of VE-cadherin. At the same time, VE-cad(1−4)$_{His}$ used in the previous study contained His tag and was detected by the reaction of this tag with the His probe (*18*), while VE-cad(1−5) was detected using anti-hVE-cadherin Ab. Thus, the lower affinity of VE-cad(1−4)$_{His}$ to $(\beta15-66)_2$ could also be attributed to the presence of His tag, the difference in detection of the bound fragments, or both. To evaluate this further, we constructed VE-cad(1−4) without His tag and studied its binding to immobilized $(\beta15-66)_2$ by ELISA using the same detection procedure as that for VE-cad(1−5). In this experiment, VE-cad (1−4) exhibited binding similar to that of VE-cad(1−5), and the determined K_d value of 39 nM was also similar (Figure 3 and Table 1). These results indicate that the affinities of VE-cad(1−5) and VE-cad(1−4) to $(\beta15-66)_2$ are very similar. They also suggest that His tag does not interfere with the fibrin-binding properties of the VE-cad(1−4)$_{His}$ fragment and that lower affinity of this fragment to $(\beta15-66)_2$ determined earlier by ELISA (*18*) may be attributed to the difference in the detection procedures.

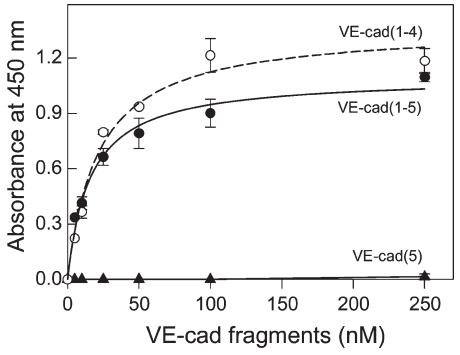

FIGURE 3: Testing of fibrin-binding properties of the fifth extracellular domain of VE-cadherin by ELISA. Increasing concentrations of the VE-cad(5) and VE-cad(1−5) fragments (filled circles and triangles, respectively) in ELISA-binding buffer were incubated with microtiter wells coated with $(\beta15-66)_2$, and the bound fragments were detected with anti-hVE-cadherin AF938 Ab, as described in Experimental Procedures. Binding of the VE-cad(1−4) fragment (open circles) to $(\beta15-66)_2$ was detected by the same procedure The curves for the VE-cad(1−5) and VE-cad(1−4) fragments (solid and broken lines, respectively) represent the best fit of the data to eq 1; the determined K_d values are presented in Table 1.

Localization of the Fibrin-Binding Site within the Third Extracellular Domain of VE-Cadherin. To further localize the fibrin-binding site of VE-cadherin, we tested interaction of the VE-cad(1−2), VE-cad(3)$_{His}$, and VE-cad(4) fragments with $(\beta15-66)_2$. In ELISA, VE-cad(3)$_{His}$ exhibited dose-dependent binding to immobilized $(\beta15-66)_2$ while VE-cad(4) failed to bind; VE-cad(1−2), which in our previous study did not exhibit any fibrin-binding activity (*18*) and was used here as a control, also failed to bind (Figure 4A). The binding of VE-cad(3)$_{His}$ occurred with high affinity (K_d = 24 nM, Table 1) and was specific, as revealed by experiments with anti-β15−21 mAb T2G-1 that efficiently inhibited this binding (Figure 4A, inset). In SPR experiments, when the $(\beta15-66)_2$ fragment at 100 nM was added to immobilized VE-cad(3)$_{His}$ or VE-cad(4), it exhibited binding only to the former (Figure 4B), in agreement with the results of ELISA. Increasing concentrations of T2G-1 mAb also inhibited this binding (Figure 4B, inset), further confirming its specificity. Altogether, these results indicate that the fibrin-binding site is located in the third domain of VE-cadherin.

To test if the neighboring domains influence fibrin-binding properties of this domain, we compared binding of the VE-cad (2−3), VE-cad(3−4), and VE-cad(2−4) fragments to immobilized $(\beta15-66)_2$ by ELISA. All three fragments exhibited dose-dependent bindings to $(\beta15-66)_2$ (data not shown) with the K_d values in the range of 27−40 nM (Table 1), i.e., similar to that for VE-cad(3)$_{His}$. These values are also similar to those determined for the interaction of $(\beta15-66)_2$ with VE-cad(1−4) and VE-cad (1−5) (Table 1). Thus, none of the extracellular domains of VE-cadherin contributes to the fibrin-binding properties of its third domain.

Further Characterization of Fibrin−VE-Cadherin Interaction by SPR. To evaluate the affinity for the interaction between the VE-cad(3)$_{His}$ and $(\beta15-66)_2$ fragments by SPR, $(\beta15-66)_2$ was added at increasing concentrations to immobilized VE-cad(3)$_{His}$, and its association/dissociation was registered

Table 1: Equilibrium Dissociation Constants (K_d) for the Interaction of Various VE-Cadherin Fragments with the $(\beta15-66)_2$ and E_1 Fragments in the Presence of $CaCl_2$ or EDTA Determined by ELISA and Surface Plasmon Resonance

	K_d, nM[b]	K_d, nM[c]		
	ELISA, $(\beta15-66)_2$	SPR, $(\beta15-66)_2$	SPR, E_1 fragment	SPR (+EDTA),[a] E_1 fragment
VE-cad(1−5)	44 ± 5	82 ± 5	79 ± 5	69 ± 30
VE-cad(1−4)	39 ± 3	80 ± 26[d]	83 ± 5	
VE-cad(1−2)	nb[e]	nb		
VE-cad(3)$_{His}$	24 ± 5	90 ± 1	93 ± 6	93 ± 13
VE-cad(4)	nb	nb		
VE-cad(5)	nb			
VE-cad(2−3)	27 ± 6			
VE-cad(3−4)	40 ± 5			
VE-cad(2−4)	32 ± 4			

[a] These experiments were performed in the presence of EDTA; all other experiments were performed in the presence of $CaCl_2$ (see text). [b] Values are means ± the standard deviation of triplicate determinations. [c] Values represent the mean of those determined by the kinetic and equilibrium analyses of the same association/dissociation data (see text). [d] Determined in the previous study with the VE-cad(1−4)$_{His}$ fragment (*18*). [e] nb, no binding observed.

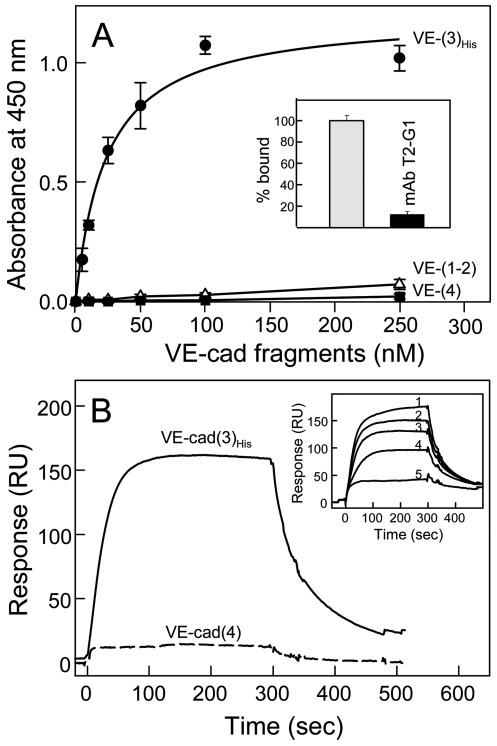

FIGURE 4: Localization of the fibrin-binding site within the third extracellular domain of VE-cadherin. Panel A: ELISA-detected interaction between the $(\beta15-66)_2$ and VE-cadhherin fragments. Increasing concentrations of the VE-cad(3)$_{His}$ (filled circles), VE-cad (4) (filled squares), or VE-cad(1−2) (empty triangles) fragments were incubated with microtiter wells coated with $(\beta15-66)_2$, and the bound fragments were detected with anti-hVE-cadherin AF938 Ab, as described in Experimental Procedures. The curve for VE-cad(3)$_{His}$ represents the best fit of the data to eq 1; the determined K_d value is presented in Table 1. Binding of VE-cad(3)$_{His}$ at 200 nM to immobilized $(\beta15-66)_2$ in the absence and presence of an equimolar amount of anti-β15−21 mAb T2-G1 (gray and black bars, respectively) is shown in the inset; the bound fragment in this case was detected with the His-tag probe. All experiments were performed in ELISA-binding buffer. Panel B: SPR-detected interaction between the $(\beta15-66)_2$ and VE-cadhherin fragments. The $(\beta15-66)_2$ fragment at 100 nM in HBS-P buffer with 1 mM $CaCl_2$ was added to immobilized VE-cad(3)$_{His}$ or VE-cad(4), and its association/dissociation was monitored in real time by registering the resonance signal (response). The inset shows binding of $(\beta15-66)_2$ at 100 nM in the same buffer to immobilized VE-cad(3)$_{His}$ in the absence (curve 1) or presence of 50, 100, 250, and 500 nM anti-β15−21 mAb T2-G1 (curves 2 through 5).

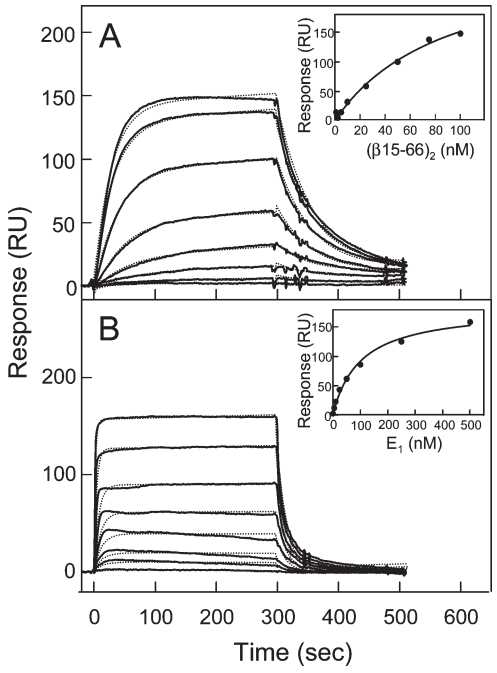

FIGURE 5: Analysis of interaction of the $(\beta15-66)_2$ and E_1 fragments with VE-cad(3)$_{His}$ by surface plasmon resonance. Panel A: $(\beta15-66)_2$ at increasing concentrations, 1, 2.5, 5, 10, 25, 50, 75, and 100 nM, was added to immobilized VE-cad(3)$_{His}$, and its association/dissociation shown by solid curves was monitored in real time. The dotted curves represent the best fit of the data using the kinetic analysis of the association/dissociation data, which gave K_d = 91 nM (Table 2); the inset shows the results of the equilibrium analysis of the same association data, which gave K_d = 89 nM; the mean K_d value (see text) is presented in Table 1. Panel B: E_1 at increasing concentrations, 1, 5, 10, 25, 50, 100, 250, and 500 nM, was added to immobilized VE-cad(3)$_{His}$, and its association/dissociation was monitored. The dotted curves represent the best fit of the data using the kinetic analysis, which gave K_d = 87 nM (Table 2); the inset shows the results of the equilibrium analysis, which gave K_d = 99 nM; the mean K_d value is presented in Table 1. All experiments were performed in HBS-P buffer containing 1 mM $CaCl_2$.

at each concentration (Figure 5A). The K_d values for this interaction determined by the kinetic and equilibrium analyses of the association/dissociation data (see Experimental Procedures) were found to be 91 nM (Table 2) and 89 nM (Figure 5A, inset), respectively. Thus, the mean K_d value of those determined by these two analyses is equal to 90 ± 1 nM (Table 1). Note that this value is more than 3-fold higher than that determined by ELISA (Table 1). At the same time, it is comparable with K_d of 80 nM determined earlier by SPR for the interaction of $(\beta15-66)_2$ with VE-cad(1−4)$_{His}$ (*18*). It is also very close to K_d for the interaction of $(\beta15-66)_2$ with VE-cad(1−5) (Table 1) determined in similar SPR experiments (not shown).

Since in the fibrin-derived E_1 fragment the VE-cadherin-binding βN-domains are in their natural environment, we also studied the interaction of VE-cad(3)$_{His}$ with this fragment. In SPR experiments similar to those described above, E_1 exhibited concentration-dependent binding to VE-cad(3)$_{His}$ (Figure 5B). The K_d values for this interaction determined by the kinetic and equilibrium analyses were found to be 87 nM (Table 2) and 99 nM (Figure 5B, insets), respectively. Thus the mean K_d value is 93 ± 6 nM (Table 1), i.e., very close to that for the interaction of VE-cad(3)$_{His}$ with $(\beta15-66)_2$. This value is also close to those determined for the interaction of E_1 with the VE-cad(1−4) and VE-cad(1−5) fragments (Table 1) in similar SPR experiments (not shown). Thus, the affinity of E_1 to these VE-cadherin fragments is very similar to that of $(\beta15-66)_2$.

It should be noted that because the conformation and adhesive properties of VE-cadherin require calcium ions (*32*, *33*), all SPR and ELISA experiments described above were performed in the presence of 1 mM $CaCl_2$ (see Experimental Procedures). Although it was reported that the addition of $CaCl_2$ or EDTA failed to either stimulate or inhibit the binding of the fibrin-derived NDSK fragment to endothelial cells and suggested that this process is Ca^{2+}-independent (*3*, *13*), no direct measurements on the influence of Ca^{2+} on fibrin−VE-cadherin interaction were performed. Thus, to test if calcium ions interfere with this interaction, we carried out SPR experiments similar to those described above; however, the binding buffer (HBS-P) used contained 2 mM EDTA instead of 1 mM $CaCl_2$. In these experiments (not shown), the E_1 fragment exhibited dose-dependent binding to both VE-cad(3)$_{His}$ and VE-cad(1−5), which was very similar to that observed in the presence of calcium ions. The

Table 2: Kinetic Parameters for the Interaction between VE-Cadherin and Fibrin Fragments

	VE-cad(3)$_{His}$			VE-cad(1−5)		
	k_{assoc} (M^{-1} s^{-1})	k_{dissoc} (s^{-1})	K_d (M)	k_{assoc} (M^{-1} s^{-1})	k_{dissoc} (s^{-1})	K_d (M)
(β15−66)$_2$	1.89×10^5	0.0172	9.10×10^{-8}	6.78×10^5	0.052	7.67×10^{-8}
E_1 fragment	7.17×10^5	0.0623	8.69×10^{-8}	16.51×10^5	0.122	7.39×10^{-8}

mean K_d values determined as above were found to be 93 and 69 nM for VE-cad(3)$_{His}$ and VE-cad(1−5), respectively (Table 1), i.e., similar to those determined in the presence of $CaCl_2$. These results indicate that calcium ions do not influence the fibrin-binding properties of VE-cadherin.

DISCUSSION

Interaction of fibrin with endothelial cells through their receptor VE-cadherin has been implicated in modulation of angiogenesis and inflammation (*3*, *5*). Our previous study characterized VE-cadherin-binding properties of the (β15−66)$_2$ fragment mimicking fibrin βN-domains and identified amino acid residues critical for its binding to VE-cadherin (*18*). In the present study, we further analyzed the molecular basis for the interaction between fibrin and VE-cadherin and found that (i) this interaction occurs exclusively through the βN-domains of fibrin, (ii) the complementary fibrin-binding site is located in the third extracellular domain of VE-cadherin, and (iii) the fibrin-binding properties of this domain are not influenced by the neighboring domains or calcium ions.

In the previous study (*18*), in which the VE-cad(1−2) and VE-cad(1−4)$_{His}$ fragments of VE-cadherin were expressed in bacteria and tested for their fibrin-binding properties, the fifth domain containing two disulfide bonds, as well as the complete extracellular portion of VE-cadherin containing this domain, was not expressed due to expected problems with proper disulfide bonding and folding of this domain. Thus, the question whether the fifth domain is involved in the interaction with fibrin remained to be addressed. That study also focused on fibrin βN-domains that were implicated in VE-cadherin binding (*3*, *18*) and did not address the question of whether other fibrin(ogen) regions/domains are involved. This question is not trivial because several cell receptors, GP IIbIIIa, $\alpha V\beta 3$, and Mac-1 (*34*−*38*), as well as some plasma proteins (*39*), interact with fibrin through its multiple binding sites, and therefore such a situation could not be excluded for the interaction of fibrin(ogen) with VE-cadherin. In the present study, we addressed these questions by preparing fibrin(ogen) fragments, which cover practically the whole fibrin (ogen) molecule, and the VE-cadherin fragments containing the fifth domain and studying interaction between them by ELISA and SPR. The results clearly indicate that the βN-domains are the only fibrin(ogen) regions involved in the interaction with VE-cadherin and that the fifth domain of VE-cadherin does not interact with fibrin.

Because the (β15−66)$_2$ fragment mimics the VE-cadherin-binding βN-domains of fibrin (*18*), this fragment was used as a probe for testing fibrin-binding properties of the VE-cadherin fragments. The affinities of its interaction with all tested fibrin-binding fragments determined by ELISA were comparable; this was also the case for the affinities determined by SPR. However, the values of K_d determined by these two methods differed by 2−3-fold (Table 1). Such differences may be attributed to the following. ELISA is a solid-phase binding assay, and K_d values determined by this assay may depend on a number of factors including conformational changes upon adsorption of proteins onto plastic surfaces, incubation time, methods of detection of bound proteins, etc. At the same time, in SPR such factors are not present, and therefore this method usually provides more accurate determination of K_d values than ELISA. This suggests that while affinities of the individual VE-cadherin fragments obtained by ELISA can be compared to each other, their comparison with those obtained by SPR may not be correct.

In this study, we also used the fibrin-derived E_1 fragment containing the βN-domains as a soluble model of fibrin. The affinity of this fragment to the VE-cadherin fragments determined by SPR was found to be very similar to that of (β15−66)$_2$ (Table 1). However, even visual comparison of association/dissociation curves for the interaction of VE-cad(3)$_{His}$ with the (β15−66)$_2$ and E_1 fragments (Figure 5) reveals obvious differences in the kinetics of these interactions. The association and dissociation rate constants, k_{assoc} and k_{dissoc}, for (β15−66)$_2$ determined from these curves were found to be different from those determined for E_1 (Table 2). Thus, although the affinities of these fragments to VE-cad(3)$_{His}$ calculated as $K_d = k_{dissoc}/k_{assoc}$ are similar (Table 2), the kinetics of their interactions are quite different. This trend was also evident in the interaction of (β15−66)$_2$ and E_1 with the VE-cad(1−5) fragment (Table 2). These suggest that the E_1 fragment, in which the βN-domains are in their natural environment, may mimic the VE-cadherin-binding properties of fibrin better than (β15−66)$_2$, which represents the isolated βN-domains.

Cell−cell adhesion through the homophilic interactions of cadherin molecules occurs only in the presence of Ca^{2+} (*40*, *32*). Previous structural studies revealed that calcium ions bind to the short linkers connecting the extracellular domains of cadherins (Figure 1B), thereby stabilizing elongated rod-like conformation of the cadherin molecule, which is important for its adhesive properties (*41*, *33*, *42*). It was also shown that in the absence of Ca^{2+} the extracellular portion of E-cadherin has a globular-like conformation, at low Ca^{2+} concentrations it adopts a rod-like structure, while higher Ca^{2+} concentrations result in homoassociation (i.e., self-association) of E-cadherin (*32*, *33*). VE-cadherin also forms rod-like structures and self-associates in a Ca^{2+}-dependent manner (*43*, *44*). In the present study, we compared binding of the E_1 fragment to the VE-cad(1−5) or VE-cad(3)$_{His}$ fragments in the presence and absence of calcium ions and found that Ca^{2+} is not required for this binding. This finding directly confirms the previous suggestion that the interaction of fibrin with VE-cadherin is Ca^{2+}-independent (*3*, *13*). It also indicates that the fibrin-binding site of VE-cadherin does not overlap with its Ca^{2+}-binding sites, located at the interfaces between the extracellular domains. This finding also suggests that the overall rod-like conformation of the extracellular portion of VE-cadherin is not required for its efficient interaction with fibrin.

The present study revealed that the interaction of fibrin with VE-cadherin occurs exclusively through the third extracellular domain of the latter. This finding narrows down the

fibrin-binding site of VE-cadherin, enabling further mapping of this site by site-directed mutagenesis and/or other techniques, and may also represent another step toward clarifying the mechanisms underlying fibrin-dependent inflammation and angiogenesis. The involvement of VE-cadherin-mediated cell−cell interactions in the control of vascular permeability (and thus leukocyte transmigration) and angiogenesis is well recognized (*20*, *22*, *45*, *46*). Although the exact mechanisms of these interactions are not established yet, several different models have been proposed based on structural studies of classical type I and type II cadherins. One of these models implicates the first two extracellular domains in homophilic interactions resulting in homoassociation of C-cadherin molecules (*42*); homoassociation of VE-cadherin was proposed to follow the same mechanism (*44*). Alternative models suggest the involvement of additional homophilic interactions through other extracellular cadherin domains (*47*−*51*). This was confirmed in recent studies, which revealed that the homoassociation of cadherins occurs in two stages and that the first two domains are required for the initial fast homophilic binding, which is weak, while the other domains, especially the third one, are required for the subsequent homophilic interaction that is characterized by slow dissociation kinetics and much stronger adhesive bonds (*52*, *53*). These studies are in agreement with the previous finding that the monoclonal antibodies directed to the first or third domain of VE-cadherin both altered endothelial cell permeability and capillary tube formation, most probably by blocking VE-cadherin-mediated homophilic adhesion through these domains (*54*). Thus, one cannot exclude that the interaction of fibrin with the third domain of VE-cadherin revealed in the present study may compete with the homophilic interaction mediated by this domain, thereby blocking VE-cadherin-mediated adhesion in the same manner. Whether such competition represents a possible mechanism involved in fibrin-dependent leukocyte transmigration and angiogenesis remains to be tested.

SUPPORTING INFORMATION AVAILABLE

Sequences of primers used to produce recombinant VE-cadherin fragments (Table S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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