The interaction of vitronectin-thrombin-antithrombin III (VN-TAT) complex with endothelial cells (EC) was investigated. Binding was specific and time- and concentration-dependent. Kinetics revealed an apparent dissociation constant of 16 nM and 1.7 x 10^5 binding sites/endothelial cell. The binding determinant of the ternary complex was located on the VN moiety. Since the association of VN to TAT adds its specific properties to the VN-TAT complex, the involvement of the heparin binding domain and the cell attachment site of VN was investigated. Neither addition of RGD peptide nor blocking of the vitronectin receptor with a monoclonal antibody interfered with VN-TAT binding to EC. Addition of heparin, a VN-derived peptide comprising two heparin binding consensus sequences or a monoclonal antibody directed against the heparin binding domain on VN, completely inhibited VN-TAT binding to EC. These results indicate that the interaction is mediated through the heparin binding domain of VN. Digestion of heparan sulfate proteoglycans resulted in a decrease of VN-TAT binding to EC, indicating the involvement of heparin-like structures on the EC surface. Our findings point to an unrecognized mechanism by which VN may act as scavenger in order to enhance the clearance of end products of the clotting system via binding of the ternary VN-TAT complex to the luminal surface of EC.

The function of the key enzyme of the blood clotting system, thrombin, as well as that of other coagulation proteases is controlled predominantly by direct interaction with serine protease inhibitors (serpins). Antithrombin III (ATIII) is considered to be the most important direct inhibitor of thrombin, and the formation of a carboxylester bond between ATIII and the active site serine of thrombin leads to an inactive, equimolar protease-inhibitor (TAT) complex. The low rate at which ATIII normally inhibits thrombin is accelerated in the presence of heparin (1). In human serum, most of the TAT complex is associated to the plasma protein, vitronectin, with which it forms a ternary complex (2, 3). Vitronectin is a glycoprotein with a molecular mass of 78,000 Da and a plasma concentration of 200–400 μg/ml (4). It is involved in several physiological processes, such as attachment of different cell types to surfaces (5), inhibition of the complement system (6), stabilization of the major inhibitor of fibrinolysis, plasminogen activator inhibitor (PAI-1) (7), and neutralization of heparin stimulation of ATIII (8). The association of VN to TAT labels the complex with the specific properties of VN, such as heparin affinity (9) and the ability to bind to cell surfaces via the RGD sequence (5). These newly generated properties of VN-TAT complex are speculated to promote a rapid disappearance of VN-TAT from the blood stream.

This prompted us to study the first event of clearance of VN-TAT, namely the interaction of the ternary VN-TAT complex with human endothelial cells, and to define the molecular characteristics of VN-TAT binding to the cell surface. Our present results demonstrate that this interaction is mediated via the heparin-binding domain of VN and heparan sulfate proteoglycan molecules on the luminal endothelial cell surface.

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemicals obtained from commercial sources were of the highest grade available. The culture plastics were purchased from Costar (Cambridge, MA). RPMI-1640 medium and the antibiotics penicillin/streptomycin and fungizone were obtained from Gibco (Biocult, Paisley, UK). The Phast-System and the fast performance liquid chromatography (FPLC) equipment were obtained from Pharmacia (Sweden). Unfractionated heparin was obtained from Organon (Oss, The Netherlands). Heparinase, heparitinase, chondroitinase ABC, and β-nitrophenyl-β-D-xylopyranoside (β-D-xyloside) were purchased from Sigma.

Polyclonal antibodies against VN and prothrombin were raised in rabbits as described by Preissner et al. (2). The polyclonal antibody against ATIII was obtained from Behring Werke (Marburg, Federal Republic of Germany). The monoclonal antibody 8E6 was kindly provided by Dr. D. Mosher (University of Wisconsin, WI). The monoclonal antibody against the β-chain of VN was a generous gift of Dr. D. Stern (Columbia Medical School, New York). RGDW peptide was a generous gift of Dr. Marguerie (Grenoble, France). Five synthetic peptides comprising the heparin binding domain of VN were kindly provided by Dr. W. Stuber (Behring Werke). TAT complex was formed by incubating α-thrombin with ATIII in a molar ratio of 1:2 for 30 min at 37°C. Purification of Proteins—VN-TAT was purified at room temperature from 100 ml of fresh human serum by affinity chromatography.
on heparin-Sepharose (50 ml, Pharmacia), equilibrated with Heps buffer (10 mM Heps, 137 mM NaCl, 0.02% sodium azide, pH 7.4). Unbound material was removed by extensive washing with Heps buffer, and bound proteins were eluted with a salt gradient from 0.15 to 1.0 M NaCl in a total volume of 500 ml of Heps buffer. The differential fractions were analyzed on 4-15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, PhastSystem) and transferred to Immobilon-P membrane (Millipore, Bradford, USA) by diffuse blotting. The blots were blocked with 5% (w/v) milk powder (Protifar, Nutricia, Holland) and incubated with polyclonal antibodies against VN, ATIII, and thrombin. The blots were washed with Tris-buffered saline (TBS-buffer; 50 mM Trizma (Tris base), 150 mM diaminobenzamidin (Sigma) in 50 ml of TBS-buffer. VN.TAT was further purified by ion-exchange chromatography on PPLC equipment. Fractions containing VN.TAT were pooled, dialyzed, against 20 mM ethanolamine (Merck, Germany), pH 9.0, and loaded on a Mono Q column. VN.TAT was eluted by a salt gradient from 0 to 1.0 M NaCl at 0.4 M NaCl in ethanolamine (20 mM, pH 9.0).

Purification of VN.TAT with a Tris buffer, pH 7.4, instead of ethanolamine, pH 9.0, did not change the characteristics of the protein (data not shown). The purified complex was kept frozen at physiological pH and remained stable.

ATIII was purified according to the method described by de Swart et al. (10). Denatured VN was purified according to the method of Yatohgo et al. (11). Purified proteins were >95% homogeneous as judged by SDS-PAGE. Modified ATIII was preincubated by incubating native ATIII with 2-fold molar excess of thrombin for 1 h at 37°C, dialyzed overnight in 20 mM ethanalamine at 4°C, and the modified form of ATIII was separated from thrombin on Mono Q (FPLC), in 20 mM ethanalamine (pH 9.0) using a salt gradient from 0 to 1.0 M NaCl.

Radiolabeling of VN.TAT—VN.TAT was labeled with [125I]NaI (Amersham, UK) using lodo-beads (Pierce Chemical Co.) and separated from free [125I]NaI by gel-filtration on Sephadex G-10 (Pharmacia), equilibrated with phosphate-buffered saline containing 0.1% bovine serum albumin (BSA, Fraction V, A-7906, Sigma). The specific radioactivity was 4–6 μCi/μg protein. The portion of free [125I]NaI was less than 4%.

Cell Culture—Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords according to the method described by Jaffe et al. (12). The culture medium contained 20% (v/v) normal human serum pool (of 20 healthy donors) in RPMI-1640 supplemented with the antibiotics penicillin (100 units/liter), streptomycin (100 μg/ml), fungizone (4 μg/ml), and sodium selenite (25 pg/ml) media supplement (Sigma). Each well contained about 20,000 EC as calculated by counting a trypsin suspension in a Rurker-Turk chamber.

Binding of VN.TAT to HUVEC—HUVEC were washed three times with Heps buffer (10 mM Heps, 137 mM NaCl, 4 mM KCl, 2 mM CaCl2, 15 mM Glucose, 5 mg/ml BSA, pH 7.4) and incubated at 4°C with 20 ng of [125I]labeled VN.TAT, diluted in a total volume of 50 μl of Heps buffer (2.5 mM). After a 2-h incubation the cells were washed five times with cold Heps buffer and the cells were lysed in 0.1 M NH4OH for 15 min at room temperature. The cell-associated label was determined by counting the lysate in a γ-scintillation counter. Measurements were performed using less than 5% recovery on two different cell batches. Representative results are shown, and binding of [125I]VN.TAT is expressed in nanograms/106 cells. To check for nonspecific VN.TAT binding to plastic, BSA-coated wells were assayed identically. Specific binding of iodinated ligand was defined as the difference in binding in the absence and presence of 100-fold molar excess of unlabeled ligand. The binding constant and number of binding sites were calculated using a computer-assisted iterative curve fit program (Ligand).

Competition assays were performed by incubating 2.5 nM radiolabeled VN.TAT in the presence of 100-fold molar excess of unlabeled compounds. The inhibitory potential of the different compounds was expressed in percentages of specific binding.

 Pretreatment of HUVEC—Preincubation at 37°C with the monoclonal antibodies anti-β, and VN27 (10 μg/ml) was performed 30 min prior to the binding assay. Non-bound antibody was washed away. Proteoglycan synthesis was inhibited by adding 2.5 mM β-D-xyloside in the culture medium 2 days before the cells were used. Glycans were incubated for 30 min prior to the binding experiment and washed away after the incubation period. The following glycans were used: heparin (5 units/ml), heparitinase (5 units/ml), chondroitin ABC (0.5 units/ml).

RESULTS

Purification of VN.TAT—Fresh human serum was used as starting material for purification of VN.TAT with heparin-Sepharose affinity chromatography. The bulk of proteins was eluted between 0.1 and 0.3 M NaCl from the heparin-Sepharose column, whereas VN.TAT eluted at 0.4–0.5 M NaCl. After further purification and concentration of VN.TAT using Mono Q ion-exchange chromatography, SDS-PAGE revealed a major protein band at 160 kDa (Fig. 1, lane 3). The protein, transferred to Immobilon-P membrane, was recognized by polyclonal antibodies against VN, ATIII, and thrombin (lanes 4–6), although anti-prothrombin reacted poorly. The immunoblots also showed bands larger than 200 kDa reacting with all three antibodies used, indicating that multimeric forms of VN.TAT were present in the eluate from the heparin-Sepharose column. Lane 1 of Fig. 1 shows an autoradiograph of [125I]VN.TAT. The mobility of labeled and unlabeled VN.TAT was identical, indicating that the labeling procedure did not modify the VN.TAT molecule.

Binding of VN.TAT to HUVEC—A confluent monolayer of HUVEC was incubated with radiolabeled VN.TAT at 4°C for the time periods indicated, in the presence or absence of 100-fold molar excess of unlabeled VN.TAT. Binding of VN.TAT to HUVEC was specific and time-dependent and reached an equilibrium within 2 h of incubation (Fig. 2). No binding was observed to albumin-coated wells. HUVEC, incubated for 1 h, were lysed in SDS-containing sample buffer and run on a 4–15% SDS-PAGE. The autoradiograph (Fig. 1, lane 2) showed that VN.TAT bound to the HUVEC did not differ from the VN.TAT before incubation (Fig. 1, lane 1), indicating the binding affinity of VN.TAT to VEC was specific and not due to nonspecific interactions. To determine whether labeling of VN.TAT altered the binding affinity to endothelial cells, confluent monolayers of HUVEC were incubated at 4°C with labeled and nonlabeled VN.TAT at varying ratios for 2 h keeping the total amount of VN.TAT constant. After measuring the cell-associated radioactivity, a linear correlation between percentage of added labeled VN.TAT and percentage of bound labeled VN.TAT was found, indicating no difference in binding affinity of labeled and nonlabeled VN.TAT (data not shown). The number and affinity of VN.TAT binding sites on HUVEC was shown, and binding of [125I]VN.TAT is expressed in nanograms/106 cells.
determined by binding of $^{125}$I-VN-TAT at 4°C as a function of VN-TAT concentration (Fig. 3). Analysis of specific binding, using a computer-assisted iterative curve fit program (Ligand), yielded an apparent dissociation constant ($K_d$) of 16 nM and $1.7 \times 10^{-7}$ binding sites per EC. In all cases the one ligand/one binding site model fitted statistically better to the binding data than the two-binding site model.

Characterization of VN-TAT Binding to HUVEC—To examine the domains of VN-TAT involved in the binding to HUVEC, competition assays were performed at 4°C. A 100-fold molar excess of urea-treated VN (VNurea), inhibited $^{125}$I-VN-TAT binding to HUVEC by 92% (Table I). Urea-treated VN was used to mimic the conformationally changed form of VN, in which it exists in the ternary complex (9). Addition of 10 μM RGDW peptide in the competition assay had no influence on the binding of VN-TAT to HUVEC. When HUVEC were preincubated with an antibody against the β3-subunit of the VN receptor, no influence on $^{125}$I-VN-TAT binding to HUVEC was found either. The RGDW peptide and the antibody against the β3-subunit were added at a concentration at which platelet aggregation, induced by ADP, was inhibited completely. Addition of 10 μM RGDW peptide did not detach the HUVEC.

The influence of heparin on the binding of $^{125}$I-VN-TAT to HUVEC is depicted in Fig. 4. Half-maximal binding was observed in the presence of 10 units/ml (100 μg/ml) unfractionated heparin, while with 300 units/ml heparin, the binding of VN-TAT to HUVEC was completely blocked. When a monoclonal antibody directed against VN (8E6) was added together with $^{125}$I-VN-TAT, complete inhibition of VN-TAT binding to HUVEC was observed (Fig. 4). This antibody was found to recognize the carboxyl-terminal region of VN, containing the entire heparin binding domain.2

Further insight into the VN binding domain involved was obtained by utilizing five synthetic peptides derived from the heparin binding region of VN (Fig. 5b). Addition of 10 μg/ml either peptide 4 or 5 inhibited the binding of $^{125}$I-VN-TAT by 40%, 10 μg/ml peptide 3 inhibited binding by 60%, whereas peptide 1 or 2 completely inhibited VN-TAT binding to HUVEC (Fig. 5a). The concentration for peptide 2 to reach half-maximal binding was 10 times lower than for peptide 1 and 3 and more than 100-fold lower then for peptides 4 and 5.

To determine which domains on the serpin- and the protease-component of the ternary complex play an additional role in VN-TAT binding to HUVEC, unlabelled IL-DIP or different forms of ATIII were added in the competition assay (Table I). IL-DIP, native (ATnat) and thrombin-modified ATIII (ATmod) did not interfere with $^{125}$I-VN-TAT binding to HUVEC, whereas preformed binary complex TAT inhibited binding by about 50%. To explain the inhibitory effect of TAT on radioabeled VN-TAT binding to HUVEC, the presence of cell surface-bound VN was taken into consideration. Preincubation of HUVEC with a monoclonal antibody, directed against VN, which was found to interfere with VN-TAT formation3 reduced the inhibitory effect of TAT on $^{125}$I-VN-TAT binding to HUVEC from 50 to 25%.

Characterization of the Binding Site Present on the HU-
peptides 1-5 within this domain are indicated.

of unfractionated heparin excess of unlabeled VN.TAT to HUVEC was performed at 4 °C for 2 h in the presence of unfractionated heparin (2.5 mM, 0.1–1000 units/ml), 100-fold molar excess of unlabeled VN-TAT (Δ), or in the presence of monoclonal antibody 8E6 (4, 10 µg/ml). Cell-associated binding was performed in triplicate and expressed in nanograms of VN-TAT bound/10⁶ cells (mean ± S.E.).

The results of the peptide competition were expressed as percentage of specific binding in the absence of peptides. The symbols used are as follows: Δ, peptide 1; ○, peptide 2; ▲, peptide 3; ●, peptide 4; ●, peptide 5. a, a portion of the primary structure of vitronectin is shown which entails the entire glycosaminoglycan binding domain.

Within this region two heparin binding consensus sequences are located, depicted as X-B-B-X-B-X and X-B-B-B-X-X-B, where B represents the probability of a basic residue and X represents a non-basic residue (17). The sequence and positions of the synthetic peptides 1–8 within this domain are indicated below the amino acid sequence.

VEC—The observation that the interaction of VN-TAT to HUVEC was mediated by the heparin binding domain of VN suggested that proteoglycans present on the EC surface are involved in this process. When HUVEC were cultured in the presence of β-d-xyloside (2.5 mM), which leads to the synthesis of glycosaminoglycan-deficient proteoglycans (13, 14), 30% inhibition of 125I-VN-TAT binding was found (Fig. 6). Treatment of the cells with heparinase for 30 min at 37 °C (5 units/ml) reduced 125I-VN-TAT binding by 40%. Hardly any influence was found when HUVEC were treated with heparitinase (5 units/ml, 30 min) or chondroitinase ABC (0.5 units/ml, 30 min). Increasing concentrations or longer incubation times did not alter these data (not shown).

**DISCUSSION**

The ultimate end products of the clotting cascade are complexes between activated proteases and protease inhibitors. Thrombin, the final enzyme of the clotting cascade, is immediately inactivated by ATIII. In human serum, thrombin-antithrombin III complexes are associated with VN (2, 3). In these ternary complexes different functional properties are combined, related to adhesive activity of VN (5) and possibly of thrombin (15) as well as potent heparin binding capacity residing in the VN component (4). Together with possible additional active site-independent mitogenic properties of thrombin (15) these complexes may provoke cell attachment, cell binding, and proliferation at the site of vessel wall injury. In the present study the interaction of ternary VN-TAT complexes with HUVEC was investigated, and the results presented here demonstrate that VN-TAT complexes bind to HUVEC in a time- and concentration-dependent manner. Binding kinetics revealed an apparent dissociation constant of 16 nM and 1.7 × 10⁵ binding sites/endothelial cell. The heparin binding domain accessible on vitronectin was responsible for the binding of VN-TAT to HUVEC, since binding was inhibited by unfractionated heparin and by a monoclonal antibody directed against the region containing the heparin binding domain of VN (8E6). Additional evidence was obtained utilizing five synthetic peptides overlapping the heparin binding domain of VN, which were able to differentially inhibit the binding of VN.TAT to HUVEC. The most efficient inhibition was found with peptide Lys148-Arg365, whose concentration needed to reach half-maximal inhibition of VN-TAT binding was 10 times lower than of peptide Ala531-Arg550 or Arg557-Arg570. This indicates that the primary binding site in VN-TAT for HUVEC is located between amino acids 348 and 361 of VN, which comprises two consensus sequences for glycosaminoglycan recognition (X-B-B-X-B) and X-B-B-B-X-B, where B represents the probability of a basic residue and X represents a non-basic residue (16). Peptide Ala341-Arg355 entails the first of the two consensus sequences, whereas peptide Arg557-Arg570 has a high homology with the second consensus sequence. The potential of these
peptides to directly bind to heparin follows the same order as found for interference with VN-TAT binding to HUVEC. No participation was found of the cell attachment site of VN during binding of VN-TAT to HUVEC. This is in agreement with Lampugnani et al. (17), who described that the VN-receptor (a,b,3) on endothelial cells is only expressed at focal contact sites (in vitro and in vivo) and is thus not available for soluble ligands.

The binding site on the endothelial cell surface for VN-TAT was characterized as proteoglycans, based on experiments in which proteoglycan processing was altered by beta-1,4-xylolide, which leads to synthesis of 50-60% glycosaminoglycan-deficient proteoglycans (14), or in which HUVEC were pretreated with heparinase. In both cases binding of VN-TAT was decreased by about 50%. Chondroitinase ABC was not effective to modify the cell surface binding site. From these experiments it is likely that heparan sulfate proteoglycans comprise the binding site on HUVEC for VN-TAT complexes. It is interesting to note that the Kd (10-40 nM) for binding of heparin to denatured VN (8) is in the same order of magnitude as the apparent Kd of VN-TAT binding for the heparan sulfate proteoglycans (16 nM) present on the cell surface. It remains to be established whether circulating VN will interfere with VN-TAT binding to HUVEC, because in vivo only a minor fraction of plasma VN is present in the heparin-binding form (18).

The two other components of the ternary complex were not directly involved in the binding of VN-TAT to HUVEC. No inhibition was found when unlabeled IL-DIP and native or thrombin-modified ATIII was added as competitor. Preformed TAT complexes, however, partly inhibited VN-TAT binding to HUVECs, although competitive interaction with heparan sulfate proteoglycans is unlikely due to the lack in heparin affinity of TAT (19). A possible explanation for the partial inhibition by TAT is that VN present at the cell surface, originating from the culture medium, may harvest TAT complexes in situ. This introduces newly formed, nonlabeled VN-TAT into the binding assay. Support for this hypothesis stems from the finding that in the presence of a VN-specific monoclonal antibody VN27, which inhibits VN-TAT formation, the inhibitory effect of TAT could partly be prevented. These findings are in agreement with Knoeller et al. (20), who found that a monoclonal antibody, recognizing the binding site on ATIII involved in the binding of TAT complex to VN, inhibited TAT binding to endothelial cells. Therefore, we conclude that epitopes present on ATIII, thrombin, or TAT are not primarily involved in binding of VN-TAT to HUVEC.

Perlmutter et al. (21) described a general serpin-enzyme complex (SEC) receptor on hepatocytes and monocytes. No information is available at present whether this receptor is also expressed on endothelial cells. Nevertheless, we added peptide 105Y, which comprises the recognition sequence of the SEC receptor, in the binding assay. Addition of peptide 105Y did not inhibit the binding of VN-TAT complex to endothelial cells (data not shown). Since we saw no residual binding of VN-TAT to HUVEC after blocking the heparin binding region of VN, we concluded that there is no participation of the SEC receptor. These findings indicate that besides a liver-dependent mechanism of TAT removal from the bloodstream, there is an endothelial cell-dependent clearance of locally formed TAT complex.

At present, ternary complexes are described for VN with three thrombin-serpin complexes, thrombin-ATIII (2, 3), thrombin-heparin cofactor II (22), and thrombin-protease nexin-I (23). Whether these three complexes compete with each other for binding to heparan sulfate proteoglycans present on the endothelial cell surface or on other cells awaits further investigation. It remains to be established as well to which extent these nonintegrin VN-receptors participate in the subsequent metabolic processing of VN-TAT complexes and to which extent these proteoglycans are involved in VN-mediated cell-matrix interactions.

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