Internalization of Vitronectin-Thrombin-Antithrombin Complex by Endothelial Cells Leads to Deposition of the Complex into the Subendothelial Matrix*

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The key enzyme of coagulation, thrombin (T),1 is rapidly inhibited by its main plasma inhibitor antithrombin (AT), with which it forms an equimolar complex. In human serum (III and Ruoslahti, 1985; Podack et al., 1986; Preissner et al., 1987) and in human plasma (de Boer et al., 1993), this binary complex associates with a third plasma protein, vitronectin (VN), resulting in the formation of a ternary vitronectin-thrombin-antithrombin (VN-TAT) complex. Upon complex formation the normally folded plasma form of VN is conformationally altered, leading to exposure of multiple domains (Tomasini and Mosher, 1988) such as a heparin-binding site and a collagen binding domain (Gebb et al., 1986). The exposure of the heparin binding domain has been found to be a prerequisite for some of the physiological properties ascribed to VN, such as binding and stabilization of plasminogen activator inhibitor (PAI-1) (Dederck et al., 1988; Wiman et al., 1988; Salonen et al., 1989) and scavenging and inactivation of the nascent C5b-9 complex of the complement cascade (Podack and Müller-Eberhard, 1979; Tschopp et al., 1988). Therefore the extended form of VN is considered to be an “activated” form of VN and TAT complex a physiological inducer of VN extension.

In a previous report we have shown that the heparin binding domain also mediates binding of VN-TAT to EC (de Boer et al., 1992). However, these experiments did not directly address the metabolic fate of VN-TAT complex bound to the endothelial cell surface. The present study provides evidence that following binding, VN-TAT is translocated through the EC and becomes deposited into the extracellular matrix. The mechanism described represents a route by which VN reaches the subendothelial matrix where it may be involved in a number of important physiological functions.

EXPERIMENTAL PROCEDURES

Materials—All chemicals obtained from commercial sources were of the highest grade available. Culture plastics (96-well plates containing 12 strips consisting of eight disconnectable wells and six-well plates) were purchased from Costar (Cambridge, MA). RPMI 1640 medium, penicillin/streptomycin, and fungizone were obtained from Gibco (Biboclt, Paisley, United Kingdom). Primarque, chloroquine, monensin, and colchicine were obtained from Sigma. Ammonium chloride was purchased from Baker (Deventer, The Netherlands) and cytchalasin B from Aldrich. Unfractionated heparin was obtained from Organon (Oss, The Netherlands).

Proteins and Antibodies—VN-TAT complex was purified from human serum as described earlier (de Boer et al., 1992). The polyclonal antibody against VN was raised in rabbits, and the IgG fraction was isolated using protein G affinity chromatography according to the instructions of the manufacturer (Pharmacia, Uppsala, Sweden). Monoclonal antibody e-ATmod directed against thrombin-modified antithrombin was kindly provided by Dr. H. Pannekoek (University of Amsterdam, The Netherlands). Fibronectin was a generous gift of Dr. J. A. van Mourik (Central Laboratory of Blood Transfusion, Amsterdam, The Netherlands).

Radiolabeling of VN-TAT—VN-TAT was labeled with [125I]Na (Amersham, United Kingdom) using IODO-BEADS (Pierce) and separated from free [125I]Na by gel filtration on Sephadex G-10 (Pharmacia), equilibrated with phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA, Fraction V, A-7906, Sigma). The specific radioactivity was 3–6 μCi/μg protein. The amount of free [125I]Na was determined by trichloroacetic acid precipitation and accounted for less than 4%.

Internalization of the ternary vitronectin-thrombin-antithrombin (VN-TAT) complex by human umbilical vein endothelial cells was investigated. Radiolabeled VN-TAT was bound to the cell surface at 4 °C, and internalization was initiated by increasing the temperature to 37 °C. After 30 min about half of the VN-TAT complex disappeared from the cell surface and accumulated in the subendothelial matrix. Translocation of VN-TAT complex from the luminal to the basolateral side was confirmed by electron microscopic evaluation of cross-sections of endothelial cells incubated with VN-TAT conjugates. Furthermore, cells cultured in VN-TAT deficient serum, incubated with purified VN-TAT, and subsequently assayed for fluorescent staining using a monoclonal antibody directed against thrombin-modified antithrombin and a polyclonal antibody against vitronectin showed co-localization of both antibodies in punctates. Punctates were randomly distributed in both the xy and xz plane of endothelial cells as evidenced by confocal laser scanning microscopy. Trichloroacetic acid precipitation and SDS-polyacrylamide gel electrophoresis showed that VN-TAT was not degraded during translocation and inhibition of the microfilament system reduced release of VN-TAT to the matrix, indicating that transcytosis was responsible for translocation. These findings emphasize that VN-TAT complex is taken up by endothelial cells, not only leading to the removal of inactivated thrombin from the circulation but also to deposition of VN into the subendothelial matrix.

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1 The abbreviations used are: T, thrombin; AT, antithrombin; EC, endothelial cell(s); ECM, endothelial cell matrix; TAT, thrombin-antithrombin; VN, vitronectin; VN-TAT, vitronectin-thrombin-antithrombin; PAI-1, plasminogen activator inhibitor; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate.
Cell Culture—Human umbilical vein endothelial cells (EC) were isolated from umbilical cords according to the method described by afe et al. (1973). The culture medium contained 20% (v/v) normal human serum pool (of 20 healthy donors) in RPMI 1640 supplemented with antibiotics penicillin (100 units/ml), streptomycin (100 μg/ml), and fungizone (4 μg/ml).

EC of the second passage were subcultured in eight-well strips (Costar) coated with fibronectin (10 μg/ml). At confluence the cells were incubated overnight with serum-free medium (RPMI 1640/penicillin/streptomycin/fungizone) with the addition of 0.5% BSA and media supplement (Sigma) containing insulin (25 μg/ml), transferrin (25 μg/ml), and sodium selenite (25 ng/ml). Each well contained about 20,000 cells. Following a 30 min preincubation a trypsin-modified antithrombin (bin-modified antithrombin) was added and the incubation temperature was raised to 37°C. At the time of the first addition inhibitors were added to the cultures 15 min prior to the binding assay and incubated for various periods of time at 37°C to allow uptake of surface-bound gold-conjugated VN-TAT complexes. Specimens were stained with 2% osmium tetroxide for overall visualization of intracellular structures. Ultrathin sections were investigated in a transmission electron microscope. The basolateral surface of cells was identified by its close proximity to the substrate, a thin coat of adhesive material attached to the bottom of the culture dish. Representative micrographs are shown.

Immunofluorescent Localization of VN-TAT—EC were cultured on glass coverslips in medium supplemented with VN-TAT-deficient serum (10% v/v), prepared by passing the serum over heparin-Sepharose. The amount of VN-TAT was checked by ELISA and accounted for less than 1%. At confluency, deficient cultures were incubated for 4 h at 37°C with deficient medium to which purified VN-TAT (5 μg/ml) was added and washed with PBS. Cells were fixed in methanol (100%) for 10 min at 20°C and co-incubated with a monospecific polyclonal antibody directed against VN and a monoclonal antibody directed against thrombin-modified antithrombin (αTAT-Thrombin) in a binding assay. Bound VN-TAT was visualized with FITC-conjugated goat anti-rabbit IgG. All antibodies were diluted in PBS containing 3% (w/v) BSA, and each step in this procedure was followed by extensive washing with PBS. Fluorescent label was applied to the first antibodies using IgG directed against mouse (FITC-conjugated) and rabbit (TRITC-conjugated) antibody (both from CLB, Amsterdam, The Netherlands). Fluorescence was analyzed using a confocal laser scanning microscope (Leitz, Heidelberg) equipped with an argon/krypton mixed gas laser and an oil-immersion objective (63× 1.4). Final images were merged using Photoshop software on an Apple computer and photographed directly from screen using a digital camera (Agfa).

Treatment of Endothelial Cells with Metabolic Inhibitors—The following inhibitors were added to serum-free medium: ammonium chloride (20 mM), chloroquine (100 μM), primaquine (100 μM), monensin (5 μM), cytochalasin B (10 μM and 400 μM), and colchicine (0.5 μM). Inhibitors were added to the cultures 15 min prior to and were present during the experiment, unless stated otherwise. After 1 h at 37°C, differentiation between internalized and matrix-deposited ligand was performed using 0.1 mM amiloride as described.

RESULTS

Time-dependent Binding and Internalization of VN-TAT Complex by EC—Binding and internalization of radiolabeled VN-TAT to ECM was preisolated by ammonia extraction at 4°C (Vöker et al., 1991). EC cultured in six-well plates were preincubated with 150 μl of the VN-TAT-gold suspension in 1 ml of serum-free medium at 4°C for 1 h and washed three times with ice-cold serum-free medium. Non-specific binding was determined by competition of the binding of the conjugate by heparin (100 units/ml). Fixation was performed with 1% formaldehyde and 0.1% glutaraldehyde in PBS. Silver-enhancement of gold-decorated EC was performed as described earlier (Vöker et al., 1991), phase-contrast illuminated in an inverted microscope, and photographed. For uptake studies, cell cultures were incubated with gold-conjugated VN-TAT at 4°C, washed as described above, and incubated for 30 min at 37°C to allow uptake of surface-bound gold-conjugated VN-TAT complexes. Specimens were stained with 2% osmium tetroxide for overall visualization of intracellular structures. Immunofluorescent localization was performed using a confocal laser scanning microscope. The basolateral surface of cells was identified by its close proximity to the substrate, a thin coat of adhesive material attached to the bottom of the culture dish. Representative micrographs are shown.

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VN-TAT by EC was investigated at 4 and 37 °C. EC were cultured on disconnectable wells and grown to confluence. Cells were incubated with radiolabeled VN-TAT (10 ng/well) over a time period of 30 min to 4 h in the absence or presence of 100-fold molar excess of unlabeled VN-TAT. The results were expressed as specific binding and uptake, which was defined as the difference in binding in the absence and presence of 100-fold molar excess of unlabeled VN-TAT complex. Data presented are representative results of three independent experiments performed in triplicate wells. Aspecific binding of radiolabeled VN-TAT on BSA-coated wells (∗) is shown in panels c and d. When symbols lack standard error bars, they are too small to extend the symbols.

To compare different extraction methods, detachment of the cells was performed with 0.1 M ammonia, 2 mM urea, or 10 mM EDTA. Removal of the cells was checked microscopically; with ammonia the cells were lysed, whereas incubation with urea or EDTA removed the cells predominantly intact (not shown). At 4 °C, 90–93% of the cell-associated label was found associated with the cell compartment, whereas 7–10% was found in the matrix. At 37 °C, 50–59% was found in the cell compartment and 41–50% was associated with the ECM (Table I).

Internalization Assay—To follow intracellular routing, radiolabeled VN-TAT (35 ng/well, 4.4 nM) was incubated with an EC monolayer for 1 h at 4 °C (designated time point 0) and cells were washed to remove non-bound material. The radioactive signal was chased by incubating the cells with an equal amount of unlabeled VN-TAT (40 ng/well). Subsequently cells were warmed to 37 °C for the time course indicated on the x axis. After 1 h of incubation at 4 °C (designated time point 0), 0.96 ng of radiolabel/well was associated with the endothelial cells (Fig. 2a). In the first 30 min of pulse-chase, about half of the initially surface-bound label was released to the medium, due to the establishment of a new equilibrium in the presence of excess of unlabeled VN-TAT complex (40 ng/well) in the medium (Fig. 2a). This amount of radiolabel in solution stayed constant during the remaining incubation period. To investigate whether VN-TAT was degraded, aliquots of the media were subjected to trichloroacetic acid precipitation. The amount of free iodine in the medium after 1 h incubation at 4 °C (time point 0) accounted for 3.9%. During incubation at 37 °C, free iodine in the media did not increase (Fig. 2b), indicating that the radiolabel was not proteolytically degraded during cellular processing. In the first 30 min at 37 °C, half of the initially surface-bound ligand was shed to the medium, indicating that the other half was bound to the cell monolayer irreversibly.

Division into cell-associated or ECM-associated label by ammonia treatment showed that after 1 h of incubation at 4 °C (time point 0) 88% was associated with the cell compartment and 12% with ECM. Raising the temperature showed that in time the cell compartment lost label, whereas ECM was enriched (Fig. 2c). To further subdivide cell-associated ligand into surface-bound and intracellular ligand, limited trypsin digestion according to Chappell et al. (1992) was performed. Surface-bound ligand was defined as ligand released from the EC-monolayer by trypsin/EDTA/proteinase K treatment. Ligand not sensitive to enzymatic digestion represented internalized

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Wells were incubated with radiolabeled VN-TAT (4.4 nM, 35 ng/well) in the absence or presence of 100-fold molar excess of unlabeled VN-TAT for 1 h at 4°C, washed, treated with ammonia, and washed. Radiolabel associated with EC monolayer before ammonia treatment, with the cell lysate, and with the matrix was measured. Defining specific binding to EC monolayer prior to ammonia treatment as 100% (a), 89% of this label was associated with the cell compartment (lys), whereas only 5% was deposited into the underlying matrix (ma, Fig. 4). Six percent of radiolabel originally associated with the cell monolayer was lost during the extraction procedure. Direct binding to preisolated matrix (PI-ma) was 23 times higher compared to matrix (ma) isolated after radiolabel incubation. The second incubation of the preisolated matrix with ammonia did not release matrix-bound label (not shown).

Electrophoretic Analysis of Internalized VN-TAT Complex—Radiolabeled VN-TAT, subjected to SDS-PAGE and autoradiography was detectable as a prominent band at 158 kDa with two additional bands, representing multimeric forms of the complex (Fig. 5, lane 1). To analyze the appearance of the complex after internalization and deposition in the matrix, cell lysates and matrices obtained by ammonia extraction were subjected to SDS-PAGE followed by autoradiography. After incubation with EC for 1 h at 4°C, radiolabeled VN-TAT was bound to the cell compartment (Fig. 5, lane 2), whereas no label was detectable in the matrix (lane 6). The composition of cell-bound label was not modified. After raising the temperature to 37°C, radiolabel disappeared from the cell compartment (lanes 3–5) and accumulated in the matrix (lanes 7–9). In all cases, matrix-deposited ternary complex retained its original molecular mass of 158 kDa.

Translocation of Gold-conjugated VN-TAT—Binding of VN-TAT complex was visualized by electron microscopic analysis in ultrathin cross-sections of EC. Cells were preloaded with gold-conjugated ternary complex for 1 h at 4°C and washed to remove non-bound material. Fig. 6a shows predominantly surface-bound gold particles organized in clusters (Fig. 6b, at higher magnification). Hardly any gold particles were associated with the basolateral side of the endothelial cells. Raising the temperature to 37°C induced internalization of the VN-TAT-gold conjugates with ultimate deposition into the subendothelial matrix (Fig. 6c). Translocation of VN-TAT-gold conjugates occurred in endosomal structures (panels c and d). Incubation of EC with gold particles alone did not lead to internalization (not shown).

Immunofluorescent Analysis of Internalized VN-TAT Complex—Internalization of VN-TAT was shown in a fluorescence experiment. EC were cultured in VN-TAT deficient serum. At confluence, cells were incubated for 3 h at 37°C with medium containing VN-TAT deficient serum to which 5 µg/ml VN-TAT was added and cells were prepared for fluorescent staining (see materials & methods). The presence of VN-TAT complex was detected with a monoclonal antibody against thrombin-modified antithrombin (αATmod) and a monospecific polyclonal antibody directed against VN. Fluorescence was visualized using confocal laser scanning microscopy. This equipment is able to scan fluorescent signals sequentially from the top of the cells into the extracellular matrix. Fluorographs can than be generated in phase (a–f) or traverse (g–l). Fig. 7 (a–f) shows en phase fluorographs, representing TAT (a–c, detected with αATmod or vitronectin (d–f). In the upper section of the cell (a and d), some fluorescent punctates are detectable but most punctates are visible in the middle section of the cell (b and e). The subendothelial matrix is diffusely stained for both TAT (c) and vitronectin (f). Distribution of punctates in traversal sections (g–l) taken at the location designated by the broken white line in
micrograph d, confirm the en phase results: punctates are visible throughout the cell compartment and associated with the extracellular matrix, which is diffusely stained. Co-localization of TAT and vitronectin is shown in a traversal detail which was scanned for TAT (FITC channel, j) and for VN (TRITC channel, k) and then the signals were merged (l). The black and white prints show identical patterns, whereas under the microscope green (FITC channel) and red signals (TRITC channel) turned into yellow signals, indicating that TAT and vitronectin co-localized in the punctates. Incubation of the cells with medium containing human serum instead of purified VN-TAT showed identical results (not shown). Cells cultured in VN-TAT deficient conditions did not show any fluorescent signal (not shown).

Treatment of the Cells with Specific Inhibitors of Endocytosis or Transcytosis—To investigate the intracellular route involved in the translocation of VN-TAT from the luminal to the basolateral side of EC, inhibitors of cellular processes were added. Ammonium chloride, chloroquine, primarque and monensin were added to the cell cultures 15 min before the label was added to the wells and were present during the binding experiment. Cytochalasin B and colchicine were only added during the binding assay, which was performed for 30 min, since longer incubation times damaged the integrity of the EC monolayer. The role of lysosomal processing was examined using NH₄Cl (20 mM), primarque (100 μM), or chloroquine (100 μM), weak bases that become concentrated in lysosomes and raise their pH (Maxfield, 1982) or monensin (5 μM), a proton ionophore which raises endocytotic vesicle pH (Harford et al., 1983). Neither of these compounds affected release of VN-TAT to the matrix (Table II). The role of transcytotic processes was investigated using cytochalasin B, which inhibits the microfilament system (Sandvig and Deurs, 1990) or colchicine, which interferes with microtubule polymerization (Sackett and Varma, 1993). Cytochalasin B (10 and 400 μM) inhibited the incorporation of VN-TAT into the matrix by 20 and 50%, respectively, and colchicine (0.5 μM) by about 25%.

**DISCUSSION**

The clearance of the equimolar thrombin-antithrombin (TAT) complex from the circulation and its distribution into extravascular tissue is coupled to ternary complex formation with a third glycoprotein in plasma, vitronectin (de Boer et al., 1993). In the present study we provide evidence for the presence of these components in the vascular wall and define requirements that lead to translocation of the luminally bound ternary complex to the basolateral side of endothelial cells (EC). Recently, we have shown that VN-TAT complex binds rapidly to EC and that the binding domain of the complex is located in the heparin binding region of the VN moiety (de Boer...
et al., 1992). Exposure of the heparin binding region occurs upon a conformational transition; native VN has no affinity for heparin. In vitro, this transition can be achieved by denaturation using chaotropes, detergent, low pH, or binding to plastic. In vivo, TAT complex may serve as a “physiological activator” of VN, since the interaction between TAT and VN leads to a similar conformational change in VN.

In the present report, we studied the destination of radiolabeled VN-TAT complex into the subendothelial matrix of human umbilical vein endothelial cells (HUVECs), cultured on glass coverslips in VN-TAT deficient medium. At confluence, cells were incubated for 3 h at 37 °C with medium to which purified VN-TAT was added. After washing, cells were fixed and permeabilized with methanol enabling the antibodies to enter the cell compartment. Internalized VN-TAT was detected with monoclonal antibody aATmod directed against thrombin-modified antithrombin and a monospecific polyclonal antibody against VN, followed by anti-mouse IgG conjugated with FITC and anti-rabbit IgG conjugated with TRITC fluorescent label. Fluorescent signals were visualized using confocal laser scanning microscopy and en phase (a–f) or traverse (g–l) micrographs were generated. Panel a–f shows en phase micrographs scanned from the top of the cell (a and d) into the subendothelial matrix (c and f) and in an intermediate section (b and e) for the presence of thrombin-modified antithrombin (FITC channel, a–c) or vitronectin (TRITC channel, d–f). In panels g–i, triplicate micrographs are shown of the part of the cell marked by the broken line in micrograph d, scanned for the presence of thrombin-modified antithrombin. Panels j–l represent a detail of some punctates scanned for thrombin-modified antithrombin (j) or vitronectin (k) and the merged signals of micrographs j and k (l), showing complete co-localization of thrombin-modified antithrombin and vitronectin. Representative micrographs are shown (amplification 63 × 1.4).
beled VN-TAT complex incubated on metabolically inactive EC (4°C) or metabolically active cells (37°C). An assay was designed that discriminates between VN-TAT associated with the cell compartment or with extracellular matrix. For this purpose endothelial cells were cultured on disconnectable wells, which could be placed in a γ-scintillation counter and measured separately. Ammonia extraction, a standard technique in our laboratory (Sixma et al., 1987), was used to lyse the cells. This method of cell removal keeps the matrix intact and firmly attached to the entire area of the culture wells (Vlodavsky et al., 1987). VN-TAT-associated radioactivity detectable in the cell lysate represented cell-associated label, whereas matrix-associated label was defined as the radioactivity left behind on the extraction method.

VN-TAT binding to EC incubated at 4°C occurred in a cell-specific, ligand-specific, time-dependent, and heparin-dependent manner, which is in accordance with previous observations (de Boer et al., 1992). EC incubated at 37°C bound VN-TAT with similar binding characteristics, but additionally radiolabel was delivered to the extracellular matrix. The appearance of radiolabeled VN-TAT in the subendothelial matrix was not due to direct binding of VN-TAT to exposed extracellular matrix, since the integrity of the monolayer remained intact during the binding assay.

This was deduced from two control experiments. (a) When matrices were preisolated by ammonia extraction and subsequently incubated with radiolabel for 1 h at 4°C, this matrix contained 27 times more radiolabel compared to the matrix isolated from an EC monolayer which had been incubated with radioligand prior to ammonia extraction. (b) Light-microscopic evaluation of EC incubated with gold-conjugated VN-TAT for 1 h at 4°C showed a fully intact EC monolayer on which radiolabel was evenly distributed.

To study internalization in more detail, a pulse-chase set-up was used in which the surface of EC was preloaded with VN-TAT at 4°C. The radioactive signal was then chased by adding unlabeled VN-TAT, and the cells were metabolically activated by raising the temperature to 37°C. In time, the cell compartment lost radiolabel, whereas the matrix was enriched with ligand. During the pulse-chase experiment, radiolabel initially bound to the cell surface was released to the medium due to the establishment of a new equilibrium in the presence of an excess amount of unlabeled VN-TAT in the medium. Radioactivity remained constant throughout the incubation period, indicating that matrix-deposited radioligand was not derived from the medium, but originated from intracellular pools. To obtain additional data on kinetics of internalization, we performed a cell removal technique using limited proteolytic trypsin degradation (Chappell et al., 1992) which discriminates between surface-bound and intracellular radioligand. We could trace an intracellular pool of radioligand; internalization reached a steady state level after 90 min of incubation at 37°C. The trypsin method detected about 10–20% less VN-TAT in the matrix compared to ammonia treatment. Apparently some VN-TAT was liberated from the matrix during the enzymatic treatment with trypsin.

The presence of an intracellular pool was also evidenced by immunofluorescent staining of endothelial cells cultured under VN-TAT deficient conditions and subsequently incubated with medium containing purified VN-TAT. Since no antibodies are available that directly recognize the ternary VN-TAT complex, cells were incubated with a monoclonal antibody directed against thrombin-modified antithrombin and a polyclonal antibody against vitronectin and double-fluorescent staining was performed. Fluorescent label co-localized in a punctated pattern randomly spread intracellularly and associated with the extracellular matrix.

These findings were complemented by electron microscopic evaluation of ultrathin sections of EC incubated with gold-conjugated VN-TAT. EM micrographs illustrated the translocation phenomenon and revealed the presence of gold conjugates in transcytotic endosomes as well as in association with the subendothelial matrix. We cannot rule out transport of VN-TAT complex via cell junctions or through lateral diffusion, although indications for this possibility could not be found in the EM micrographs.

Transcytosis rather than endocytosis seemed to be involved in the translocation of the complex from the luminal to the basolateral side of the EC as was concluded upon several observations. (a) Endosomal structures were involved in the translocation from the luminal to the basolateral side of the EC. (b) Electrophoretic analysis of matrix-deposited complex showed that the complex was released to the matrix fully intact. (c) Trichloroacetic acid precipitation of media collected during the pulse-chase experiment showed a constant level of free iodine, indicating that VN-TAT was not degraded during internalization. (c) Specific inhibitors of lysosomal function and endocytosis such as ammonium chloride, chloroquine, pramquine, or monensin did not influence matrix deposition, whereas specific inhibitors of transcytosis such as cytochalasin B and colchicine partially decreased matrix deposition. It should be noted that treatment with cytochalasin B and colchicine, which may induce cell death in time, was carried out only for a short period of time in order to minimize loss of integrity of the EC monolayer. This may explain their relatively mild effect on matrix deposition compared to the high concentrations used.

Various plasma molecules cross the endothelium by receptor-mediated transcytosis. For instance, insulin (King and Johnson, 1985) as well as some carrier proteins like albumin (Ghi tescu et al., 1986), transferrin (Jefferyes et al., 1984), or lipoprotein lipase (Saxena et al., 1990) are transported through the endothelium very efficiently without degradation. Especially the processing of lipoprotein lipase by endothelial cells is of interest, since striking similarities are apparent compared to VN-TAT; binding is heparin-dependent, internalization reaches a steady state level (Saxena et al., 1990), and during internalization no degradation occurs. Translocation of lipoprotein lipase is decreased in the presence of cytochalasin B but not affected by chloroquine, indicating that transcytosis is involved as well. The establishment of a steady state level of internalized ligand may be characteristic for transcytosis and
may be due to very rapid translocation of ligand. In the case of VN-TAT, label appeared in the matrix almost immediately and exceeded the amount of intracellular ligand.

When incubated on fibroblasts, lipoprotein lipase is internalized and subsequently degraded through a lysosomal pathway (Chappell et al., 1992). This was shown to occur via the low density lipoprotein receptor-related protein pathway.

Lipoprotein receptor-related protein cannot be responsible for internalization of VN-TAT by endothelial cells, since HUVEC do not express this receptor (Godyna et al., 1995). Interestingly, fibroblasts are able to process and degrade conformationally altered, non-complexed VN (Panetti and McKeown-Longo, 1993). This was shown to be mediated by the $\alpha_v$ integrin receptor. This receptor cannot be involved in the above described pathway either, since RGD peptides have no effect on binding of VN-TAT to HUVEC (de Boer et al., 1992). Recently Waltz and Chapman (1994) showed that the urokinase plasminogen activator receptor binds conformationally altered VN. Furthermore, fibroblasts are its abilities to promote cell attachment and to stabilize PAI-1, a major inhibitor of plasminogen activation. Purified VN-TAT complex is still able to bind PAI-1 and promote cell attachment, indicating that the ternary complex may fulfill crucial functions at its final destination in the vessel wall.

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