Annexin V Inhibits the Procoagulant Activity of Matrices of TNF-Stimulated Endothelium Under Blood Flow Conditions

Waander L. van Heerde, Kjell S. Sakariassen, H. Coenraad Hemker, Jan J. Sixma, Chris P.M. Reutelingsperger, Philip G. de Groot

Abstract A human ex vivo thrombosis model was used to investigate whether recombinant annexin V (rANV) can prevent thrombus formation under venous and arterial blood flow conditions. In this model, blood from an antecubital vein of healthy donors was allowed to flow directly over the extracellular matrix of tumor necrosis factor–stimulated endothelial cells (TNF-ECMs). TNF-ECMs were preincubated with rANV (2.9 μmol/L) for 30 minutes. With this rANV concentration all binding sites present on TNF-ECMs (1.6±0.5 x 10^12/cm^2) are occupied, and a maximal inhibition was observed in a tissue factor–dependent clotting assay. Fibrin deposition and platelet and leukocyte adhesion were measured on the rANV-treated and nontreated TNF-ECMs. Nontreated TNF-ECMs were used as controls. rANV inhibited fibrin deposition by 81% at a wall shear rate of 100 s^-1. A nonsignificant inhibition was also observed at 650 s^-1. Platelet-matrix adhesion, which is more prominent at higher shear rates, was significantly decreased by 60% at 100 s^-1 but not at 650 s^-1. The average leukocyte adherence was nonsignificantly lowered at 100 s^-1. Virtually no leukocytes adhered at 650 s^-1. The results demonstrated that rANV can inhibit blood coagulation under venous blood flow conditions and may serve as an antithrombotic drug. (Arterioscler Thromb. 1994;14:824-830.)

Key Words • annexin V • blood flow • endothelial cell matrix • ex vivo studies

ubendothelial structures become exposed to the circulating blood through damage of the vessel wall. Exposed subendothelium is highly thrombogenic because tissue factor and proteins adhesive for platelets are present.1-3 Ryan et al4 report that tissue factor is associated with phospholipid vesicles in tumor necrosis factor–stimulated endothelial cell matrices (TNF-ECMs).

The protein annexin V has recently been identified, purified, and cloned. Annexin V has been detected in a wide variety of tissues and intracellularly in different cell types5-6 and binds with high affinity to negatively charged phospholipids.7-9 These phospholipids are important at different stages of blood coagulation, and as a consequence of its phospholipid-binding properties, annexin V inhibits blood coagulation in vitro.7,8,10-15 Annexin V belongs to a multigene family of calcium-dependent phospholipid binding proteins consisting of at least 13 different members.16-19 They all share functional and structural properties (for reviews see References 20 through 23). In addition to the anticoagulant property of annexin V, other in vitro activities include inhibition of protein kinase C activity,24 inhibition of phospholipase A_2 activity,25 and formation of voltage-dependent Ca^{2+} channels.26 Despite all these in vitro studies, the physiological function of annexin V in vivo remains to be clarified.

We addressed the question of whether recombinant annexin V (rANV) can inhibit the hemostatic process under blood flow conditions in humans. Studies were performed using a human ex vivo thrombosis model in which nonanticoagulated blood was perfused directly over TNF-ECMs.27 TNF-ECMs were preincubated with rANV to investigate the inhibitory capacity of rANV on fibrin and platelet deposition. As a control, nontreated TNF-ECMs were used. To determine the optimal conditions for blood perfusion experiments, binding and coagulation inhibition studies were performed first with rANV.

Methods

Materials

All plastic materials for cell culture were obtained from Nunc or Costar. All other tissue-culture supplies (media, antibiotics, trypsin) were obtained from Gibco Biocult. Lyophilized crude human brain thromboplastin (tissue factor) was a gift from Tore Jansen, Nycomed Pharma. The monoclonal antibody (Mab) against tissue factor, Mab HTF1-7B8, was a gift from Dr Yale Nemerson. Thermax coverslips were purchased from Flow Laboratories. Fluorescein isothiocyanate (FITC) was obtained from Sigma Chemical Co. All other reagents were of the highest grade commercially available.

Cell Culture and ECM Preparation

Human umbilical vein ECs were isolated and cultured as described28 with some minor modifications.29 For binding studies of rANV to ECMs, second-passage ECs were subcultured on fibronectin-coated 96-well tissue-culture plates (0.33 cm^2/well). Cell monolayers grown to confluence in 5 through 7 days were used (approximately 6 x 10^5 cells/cm^2). At confluence, culture medium was refreshed approximately 16 hours before starting the experiments. To induce tissue factor expression, ECs were stimulated with 300 pmol/L TNF for 4 hours and 30 minutes. To isolate matrices, cells were removed by incubation with 0.1 mol/L NH_4OH for 5 minutes. Matrices

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were extensively washed with phosphate-buffered saline (PBS) and stored in freshly prepared PBS at 4°C for 1 day before they were used in binding studies.

Ex vivo perfusion studies were performed with TNF-ECMs cultured on Thermannox coverslips. ECs of the second passage were subcultured on gelatin-coated Thermannox coverslips. Before seeding the ECs, gelatin on the coverslips was fixed with 0.5% glutaraldehyde. Cell monolayers grown to confluence in 5 through 7 days were used (approximately 6x10^6 cells/cm²). Sixteen hours before stimulation of the cells, fresh culture medium was added. To induce tissue factor expression, ECs were stimulated with 300 pmol/L TNF for 4 hours and 30 minutes. Nonstimulated ECs were similarly processed, but no TNF was added. The matrices were subsequently isolated as described above.

Fluorescence studies were performed with ECs cultured on glass coverslips. The culture procedure was the same as on Thermannox coverslips. For tissue factor determination, confluent ECs were stimulated with 300 pmol/L TNF-aff for 4 hours and 30 minutes were scrapped from the bottom of the culture flask with a rubber policeman (cell scraper, Costar) in the presence of a buffer containing 130 mmol/L NaCl, 2 mmol/L KCl, 5 mmol/L CaCl_2, 12 mmol/L NaHCO_3, pH 7.4, 2.5 mmol/L MgCl_2, and 0.9 mmol/L HEPES buffer containing 5 mmol/L CaCl_2. Nonspecific binding was observed in the presence of 14 mmol/L nonlabeled rANV. The incubation was stopped by washing the wells three times with HEPES buffer containing 5 mmol/L CaCl_2. The glass coverslips were analyzed by using a Reichert-Jung Polyvar light microscope (Leica). Photomicrographs were taken in the fluorescent mode.

**Inhibition of Tissue Factor–Dependent Coagulation by rANV**

The capacity of rANV to inhibit the procoagulant activity of EC homogenates or crude human brain thromboplastin (tissue factor) was determined by a modified one-stage clotting assay. Briefly, 50 µL pooled human citrated plasma and 50 µL buffer C containing different concentrations of rANV (0 to 5 µmol/L) were added to a 50-µL homogenate of ECs stimulated with TNF or to crude human brain thromboplastin diluted 10 times in buffer C. After 3 minutes' preincubation, the assay was started by adding 50 µL prewarmed CaCl_2 (25 mmol/L), and clotting times were measured. Procoagulant activity was expressed in arbitrary units of tissue factor activity determined by reference curves obtained with the EC homogenate or thromboplastin. Reference curves (log tissue factor dilution versus log clotting time plots) from fractions of the TNF-stimulated EC homogenate or thromboplastin diluted in buffer C were linear over the range of clotting times studied. To study the effect of rANV on homogenized TNF-stimulated ECs or on thromboplastin procoagulant activity, clotting times were measured and the remaining tissue factor activity was calculated using the corresponding reference curves. The tissue factor activity measured without any addition of rANV was defined as 100% activity. The tissue factor activity was defined as 0% in the presence of Mab HTF1-7B8.

**Blood Donors**

Nonanticoagulated blood for ex vivo perfusion experiments was donated by 24 healthy volunteers who denied having used drugs during the last 10 days before the donation. Fifty-five milliliters of blood was drawn from an antecubital vein of each donor with a No. 19 butterfly infusion set (Abbott Laboratories). The first 5 mL was collected into EDTA to measure the hematocrit and platelet count. The remaining 50 mL was used for the ex vivo perfusion experiment. Platelet counts (1.4 to 4.4x10^11/L) and hematocrit (37% to 49%) of all donors were within the normal range.

**Ex Vivo Perfusion Experiments With Nonanticoagulated Blood**

For ex vivo perfusion experiments, Thermannox coverslips containing TNF-ECMs were preincubated with 2.9 µmol/L rANV in buffer C for 30 minutes at room temperature. The coverslip was positioned in a prewarmed (37°C) parallel-plate perfusion chamber. Blood from an antecubital vein of healthy individuals was allowed to flow directly into the chamber. The blood flow rate was 10 mL/min, which in chambers having different geometrical dimensions resulted in wall shear rates of 100 s⁻¹ and 650 s⁻¹. These wall shear rates reflect blood flow conditions for small veins and medium-sized arteries, respectively. The blood perfusions were followed by a
20-second perfusion with buffer C and a 40-second perfusion with a buffer containing 0.1 mol/L cacodylate, 2.5 mmol/L CaCl₂, 0.9 mmol/L MgCl₂, and 2.5% (vol/vol) glutaraldehyde. Immediately after perfusion, the coverslip with the TNF-ECMs and blood deposits was removed from the chamber and postfixed in the same buffer. The coverslips were stored in a buffer containing 0.1 mol/L cacodylate and 7% (wt/vol) sucrose and finally embedded in Epon.

Morphometry of Fibrin and Platelet Deposition

Semithin sections of 1 μm thickness for light microscopic morphometry of platelet-surface and fibrin-surface interactions were prepared from Epon-embedded preparations at an axial position of 1 mm downstream from the flow inlet and perpendicular to the direction of blood flow. The sections were stained with toluidine blue and basic fuchsin and analyzed by standard morphometry. This procedure was used to differentiate and quantify the percent surface coverage with contact platelets (platelets not spread out on the surface), spread platelets, fibrin, thrombi more than 2.5 and 5.0 μm in height, and leukocytes. All results were expressed as percentage surface coverage except for leukocyte adhesion, which was evaluated as number of leukocytes per 100 μm sectional length and expressed as number of leukocytes per 100 μm.

Statistical Analysis

Significance of the unpaired data was calculated with the multiple ANOVA (MANOVA) procedure. Values of P<.05 were considered significant.

Results

To perform the ex vivo perfusion studies under optimal conditions, we first investigated the binding capacity of rANV to extracellular matrices of quiescent and TNF-stimulated ECs and the inhibitory capacity of rANV in a tissue factor–dependent one-stage clotting assay. The ex vivo perfusions were performed with an rANV concentration that would occupy all rANV binding sites on the matrices and cause maximal inhibition in the tissue factor–dependent clotting assay.

Binding of rANV to ECMs

To determine whether radiolabeling altered the binding properties of rANV to ECMs, ECMs were incubated at 4°C for 1 hour with mixtures of labeled and nonlabeled rANV at varying ratios; rANV concentration was held constant (71 nmol/L) to cause saturated binding. A linear correlation (r = .992) was found between the percentage of labeled rANV bound to ECMs and the percentage of nonlabeled rANV added with a slope of −1.0, indicating that nonlabeled and labeled rANV compete with identical affinities for the same ECM binding sites (results not shown). A similar experiment was performed with mixtures of 125I-rANV and FITC-labeled rANV. No differences were observed, indicating that fluorescent labeling did not affect the binding characteristics of rANV to ECMs.

Matrices of quiescent and TNF-stimulated ECs were incubated with 125I-rANV at 4°C for different time periods. Equilibrium of binding was reached within 5 minutes and was stable for at least 2 hours. No difference in the total amount of rANV bound to matrices of quiescent and TNF-stimulated ECs was observed. Storage of the matrices at 4°C for 1 week did not influence the binding characteristics of rANV to either matrix. Nonspecific binding was less than 5% of the total binding in the presence of a 400-fold molar excess of nonlabeled rANV or when matrices were isolated with 0.5% Triton X-100 (data not shown).

Number of binding sites per square centimeter and kₐ of rANV on quiescent ECMs and TNF-ECMs were determined by incubating the matrices with various concentrations of 125I-rANV. The result of a typical binding experiment performed on matrices isolated from quiescent ECs is shown in Fig 1. All results were corrected for nonspecific binding measured with 125I-rANV in the presence of a 400-molar excess of nonlabeled rANV. In all cases, nonspecific binding was less than 5% of the total binding. Binding of radiolabeled rANV to ECMs was saturable; the same results were obtained with TNF-ECMs. Binding parameters calculated using the nonlinear data-fitting program ENZFITTER are shown in Table 1.

The results presented in Table 1 were performed in 96-well plates. No differences in binding characteristics were observed when binding studies were performed on Thermanox coverslips containing ECMs or TNF-ECMs. Furthermore, 125I-rANV did not bind to coverslips lacking ECMs. Changing the calcium concentration from 5 to 3 mmol/L did not affect the binding characteristics.

### Table 1. Dissociation Constants and Number of Binding Sites of rANV to Quiescent and TNF-Stimulated ECMs

<table>
<thead>
<tr>
<th>ECM</th>
<th>Binding Sites/cm² (x10⁶)</th>
<th>kₐ, nmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECM</td>
<td>1.8±0.8</td>
<td>2.7±1.0</td>
</tr>
<tr>
<td>TNF-ECM</td>
<td>1.6±0.5</td>
<td>2.7±1.1</td>
</tr>
</tbody>
</table>

The dissociation constant (kₐ) and number of binding sites were measured by incubating matrices of quiescent endothelial cells (ECM) and tumor necrosis factor–stimulated ECMs (TNF-ECM) for 1 hour with different concentrations of radiolabeled recombinant annexin V (rANV). Radioactivity was counted after the matrices were washed. Results are mean±SD (n=6).
FIG 2. Photomicrographs showing binding of recombinant annexin V–fluorescein isothiocyanate (rANV-FITC) to matrices of nonstimulated endothelial cells (ECMs). A, ECMs were incubated with 70 nmol/L rANV-FITC for 1 hour at 4°C. B, Nonspecific binding was observed in the presence of a 200-fold excess of nonlabeled rANV. The binding procedure was performed as described in "Methods." Bar=22.5 μm.

Fluorescence studies were performed with FITC-rANV on matrices of quiescent ECs. Fig 2A shows a typical granular staining pattern for FITC-rANV on the matrix. Fluorescence was completely abolished by an excess of nonlabeled rANV (Fig 2B).

rANV-Induced Inhibition of the Tissue Factor-Dependent Clotting Assay

Tissue factor–dependent coagulation was studied in a modified one-stage clotting assay. Homogenates of TNF-stimulated ECs or crude human brain thromboplastin were used as a source for tissue factor. Addition of Mab HTF1-7B8 prolonged the tissue factor–dependent clotting time for TNF-stimulated homogenized ECs from 40 to 97 seconds and for thromboplastin (tissue factor) from 21 to 100 seconds, respectively. Increasing the concentration of Mab HTF1-7B8 did not further prolong the clotting time.

The effects of various rANV concentrations on the clotting time in the modified one-stage clotting assay were measured for both tissue factor sources (Fig 3). All experiments are expressed in percentage tissue factor activity. Clotting times measured in the presence of Mab HTF1-7B8 were considered as 0% activity. Normal clotting times, without addition of an antibody against tissue factor, were expressed as 100% activity. Coagulation induced by extracts of homogenized TNF-stimulated ECs was inhibited by 100% at an rANV concentration of 200 nmol/L according to the reference curves obtained with the same extract. Half-maximal inhibition (IC50) was observed at 19±6.0 nmol/L rANV. On the other hand, more than 95% inhibition of the crude human brain thromboplastin (tissue factor)–induced coagulation assay was reached at approximately 2 μmol/L rANV. The IC50 value for this assay was 160±10 nmol/L.

Ex Vivo Perfusion Experiments

To study the effect of rANV on hemostatic and thrombotic processes in a more physiological system, human ex vivo perfusion experiments were performed with TNF-ECMs at wall shear rates of 100 s⁻¹ and 650 s⁻¹. Before exposure to blood flow, TNF-ECMs were preincubated with 2.9 μmol/L rANV, a concentration that was 1000 times the kₐ. This concentration was chosen to ensure that all rANV binding sites were occupied. Furthermore, the 2.9 μmol/L rANV concentration was 10 times higher than the concentration needed to inhibit TNF-stimulated EC extract–induced clotting by 100%. Blood TNF-ECM interactions were differentiated and quantified as fibrin deposition (Fig 4A), platelet adhesion to TNF-ECM and fibrin (Fig 4B), and leukocyte adhesion to TNF-ECM and fibrin (Fig 4C).

TNF-ECMs were highly procoagulant and induced an average surface coverage with fibrin of 73±6% and 15±11% at wall shear rates of 100 s⁻¹ and 650 s⁻¹, respectively (P=.001; Fig 4A). Preincubation of the TNF-ECMs with rANV reduced the fibrin deposition by 81% (from 73±6% to 14±6%) at a shear rate of 100 s⁻¹ (P<.001). Fibrin deposition at 650 s⁻¹ was nonsignificantly reduced by rANV by 83%, from 15±11% to 3±1%.

Platelet adhesion to TNF-ECMs (Fig 4B) was evaluated on the same sections used to quantify the fibrin deposition. Platelet adhesion included morphologically identified contact platelets, spread platelets, and spread platelets at the base of thrombi. At a wall shear rate of 650 s⁻¹, platelet adhesion to fibrin and ECMs was
FIG 4. Bar graphs showing effects of recombinant annexin V (rANV) on fibrin deposition (A), platelet adhesion (B), and leukocyte adhesion (C). Tumor necrosis factor-stimulated endothelial cell matrices (TNF-ECMs) were perfused with nonanticoagulated blood at wall shear rates of 100 s⁻¹ and 650 s⁻¹. The values are mean±SEM (6 experiments) obtained with nontreated TNF-ECMs (closed bars) or rANV-treated TNF-ECMs (hatched bars). **P<.001, *P<.05.

Platelet adhesion at 650 s⁻¹ (50±5% versus 58±10%). The respective values for surface coverage with contact platelets, spread platelets, and thrombi are shown in Table 2. Spread platelets predominated at 100 s⁻¹. At 100 s⁻¹ fewer platelets adhered to the rANV-preincubated matrices and a significant shift from spread to contact platelets was observed (P<.05). At 650 s⁻¹, significantly more platelet thrombi were observed than at a wall shear rate of 100 s⁻¹ (P<.005). Preincubation of TNF-ECMs with rANV at 650 s⁻¹ failed to significantly decrease the average surface coverage with platelets and thrombi.

Leukocyte adhesion to TNF-ECMs and to fibrin was studied on the same sections. Leukocyte adhesion at 100 s⁻¹ was significantly higher than at 650 s⁻¹ (5.6±1.4 versus 0.2±0.1 leukocytes/100 μm; P<.005). At 100 s⁻¹, a nonsignificant 64% decrease was observed when TNF-ECMs were preincubated with rANV (2.1±1.1 leukocytes/100 μm). No effect of rANV was observed at 650 s⁻¹, but the number of adherent leukocytes in the control situation was so low that further reduction could hardly be achieved.

**Discussion**

Damage of the luminal surface of the vessel wall leads to thrombus formation on the exposed subendothelial structures. Thrombus formation is the result of an intricate interplay between platelets and the humoral coagulation system. Thrombin plays a pivotal role in this process because it activates platelets and zymogens of the blood coagulation system, leading to fibrin formation. A new, promising possibility to prevent unwanted thrombin formation is to reduce the amount of negatively charged phospholipids necessary to support the formation of clotting factor complexes. This can be achieved with annexin V, which has a higher affinity for negatively charged phospholipids than the vitamin K-dependent clotting factors.8,13 A very low concentration of annexin V can inhibit the formation of thrombin in in vitro clotting assays.17,12,15 More Information on whether annexin V can inhibit thrombus formation in flowing human native blood has previously been unavailable; the only in vivo studies were performed in animals.37,38 Here, we studied the influence of the addition of rANV on fibrin deposition and platelet adhesion in a human ex vivo thrombosis model in which nonanticoagulated blood was perfused over TNF-ECMs at two different wall shear rates, 100 s⁻¹ and 650 s⁻¹.

| TABLE 2. Platelet-Matrix/Fibrin Interactions After Perfusions Over Nontreated and rANV-Treated Tumor Necrosis Factor-Stimulated Endothelial Cell Matrices |
|---|---|---|
| Shear Rate | 100 s⁻¹ | 650 s⁻¹ |
| Adhesion | Control | +rANV | Control | +rANV |
| Contact platelets, % | 0±0 | 1.7±0.4 | 0.2±0.1 | 0.8±0.3 |
| Spread platelets, % | 18±4 | 6.0±1.4 | 40±7 | 51±9 |
| Thrombi of 2.5-5 μmol/L, % | 0.3±0.2 | 0±0 | 6.1±1.4 | 4.6±2.8 |
| Thrombi of >5 μmol/L, % | 0.3±0.3 | 0±0 | 3.8±1.1 | 1.7±1.5 |
| Total platelet adhesion, % | 19±4 | 8±1 | 50±5 | 58±10 |

Effect of recombinant annexin V (rANV) on platelet-matrix/fibrin interactions after perfusions of tumor necrosis factor-stimulated endothelial cell matrices at wall shear rates of 100 s⁻¹ and 650 s⁻¹. Results are mean±SEM (n=6) of the respective surface coverages.
These wall shear rates reflect blood flow conditions in small veins and intermediate-sized arteries, respectively. To study the effect of rANV, TNF-ECMs were preincubated with 2.9 μmol/L rANV for 30 minutes, conditions that would lead to occupation of all binding sites and complete inhibition of tissue factor–dependent coagulation under static conditions. However, it is not possible to mix rANV with the blood in the perfusion system before it is perfused over the TNF-ECM without the introduction of substantial activation of platelets and coagulation. Furthermore, no toxicity studies are available in animals, and administration of rANV to human volunteers is not allowed. TNF-ECMs were used as a model for subendothelium. Before harvesting the matrices, ECs were stimulated with TNF to induce tissue factor synthesis. Tissue factor synthesized by TNF-stimulated ECs is mainly deposited in phospholipid vesicles in the ECM. To detect maximal effects of rANV in the ex vivo perfusion study, we first investigated whether rANV could inhibit EC-mediated activation of coagulation, and we determined the binding characteristics of rANV to quiescent ECM and TNF-ECM.

Characterization of the interaction of rANV with matrices of quiescent and TNF-stimulated ECs showed that rANV binds with a Kd of 2.7±1.0 mM. Assuming that each rANV molecule covers 2.7±10⁻³ m², 40% of the total surface is covered with rANV. In binding studies performed with FITC-labeled rANV, a granular staining pattern was observed, but when binding studies were performed with matrices isolated with Triton X-100, no binding of ³⁵S-rANV could be observed. This suggests that phospholipids are the rANV binding sites in matrices of quiescent and TNF-stimulated ECs. These phospholipids are probably present in vesicle-like structures in the matrix that also contain tissue factor, the initiator of blood coagulation. The Kd for binding of rANV to intact ECs was approximately five times higher (16±3 mM), and the number of binding sites present on intact ECs was about threefold less than on matrices of quiescent and TNF-stimulated ECs. A possible explanation for this difference between intact cells and ECM is that other types and/or other ratios of phospholipids are present in ECM. Stimulation of the ECs with TNF apparently did not change the amount and composition of negatively charged phospholipids in ECM. Subtle changes in the phospholipid composition cannot be excluded, because one annexin V molecule covers about 42 phospholipids, of which at least two need to be negatively charged to mediate annexin V binding.

rANV inhibited fibrin deposition in the human ex vivo thrombosis model by more than 80% at both shear rates tested. Only the results at 100 s⁻¹ were significant, apparently due to the already relatively low fibrin deposition in the absence of rANV at 650 s⁻¹. Van Ryn-McKenna et al. studied the inhibition of fibrin deposition on injured jugular veins in vivo by high concentrations of annexin V in a rabbit model. Comparable results were found for fibrin deposition, indicating that no differences were present between preincubating the injured vessel wall with annexin V, as we did, or adding it to the circulation, as Van Ryn-McKenna et al. did. However, in the latter study no information was presented about platelet adhesion and platelet thrombus formation.

The inhibition of platelet adhesion at 100 s⁻¹ is probably due to the decreased amount of fibrin deposition on the surface, since fibrin formed on the ECM is probably a major adhesive protein for platelets under low shear conditions. At 650 s⁻¹ no effect of rANV was observed on platelet adhesion, indicating that decreased α-thrombin formation had no effect on platelet adhesion, which is consistent with previous results. On the other hand, Zwaginga et al. and others reported that α-thrombin does affect thrombus formation. We also found diminished thrombus formation when ECs were preincubated with rANV at 650 s⁻¹. However, this difference was small and not significant. Collagen in the ECM can also induce thrombus formation. Moreover, we preincubated the ECM with rANV. Initial thrombin formation on rANV-treated ECs was reduced, but later thrombin generation could occur on surfaces exposed by activated platelets. After rANV treatment, a significant shift from spread platelets to contact platelets was seen at 100 s⁻¹. The same shift was observed at 650 s⁻¹, but this was not significant. Lack of platelet spreading is seen in Glanzmann’s thrombasthenia and with anti-glycoprotein IIb/IIIa antibodies. For this reason, it is generally assumed that glycoprotein IIb/IIIa is involved in platelet spreading. rANV treatment evidently leads to less activation of glycoprotein IIb/IIIa.

Perfusion studies did not show a direct effect of rANV on platelet activation, which contrast with experiments performed in vitro in which rANV was capable of inhibiting the release of 12-hydroxy-(5Z, 8Z, 10E, 14Z)icosatetraenoic acid and thromboxane B₂ by thrombin by 40%. However, the concentrations used were approximately 20 times higher than the concentration used in this study.

We conclude that rANV is a potent inhibitor of thrombus formation under venous blood flow conditions and that it may therefore serve as a possible antithrombotic drug candidate for deep venous thrombosis.

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References

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