Recombinant Annexin II Modulates Impaired Fibrinolytic Activity In Vitro and in Rat Carotid Artery

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Abstract—Fibrinolytic activity has been reported to be decreased in atherosclerosis. Recently, annexin II was identified as a coreceptor on endothelial cells for plasminogen and tissue plasminogen activator. In this study, we examined whether recombinant annexin II (rAN II) protein can modulate fibrinolytic activity on vascular endothelium in vitro and in vivo.

The effect of rAN II on human umbilical vein endothelial cells (HUVECs) was measured. Addition of a fluorescent plasmin substrate revealed that HUVECs treated with rAN II exhibited significantly more plasmin generation than those treated with BSA. Moreover, rAN II treatment of HUVECs restored plasmin generation impaired by plasminogen activator inhibitor-1 or homocysteine pretreatment. In a rat carotid artery thrombus model, the patency of thrombosed carotid arteries was significantly enhanced by rAN II injection, in contrast to BSA injection, without systemic blood coagulation dysregulation. We found that rAN II enhanced plasmin generation on vascular endothelium in vitro and reduced thrombus formation in vivo, and concluded that enhancement of endothelial fibrinolytic activity by annexin II could modulate the hypercoagulable state of atherosclerosis. Further study of rAN II in vitro and in vivo may lead to the establishment of novel therapeutic approaches to thrombogenic vascular disease. (Circ Res. 2001;89:1240-1245.)

Key Words: annexin II ■ rat carotid artery ■ thrombus

Vascular endothelial cell luminal surfaces maintain a “thromboresistant status” by dynamic balancing between the coagulation and fibrinolytic systems; however, the damage to vessel walls often seen in ischemic syndromes leads to thrombus formation. The fibrinolytic characteristics of vascular endothelium are modulated by the activity of plasminogen activators that facilitate conversion of plasminogen to active plasmin,1 and the binding of plasminogen to endothelial cells significantly enhances the catalytic efficiency of its activation by tissue plasminogen activator (t-PA).2,3

Annexins are a superfamily of calcium-dependent phospholipid binding proteins,4 which have a highly conserved core domain preceded by a more variable amino terminal tail domain.5 Recently, annexin II, a member of this superfamily, has been identified on endothelial cells and found to bind t-PA and plasminogen, as well as to enhance plasmin generation.6,7

The expression and cell surface transport of annexin II is not fully understood; however, several important features of this molecule have been reported, including calcium-dependent high-affinity binding to phospholipid.5,9 Annexin II is now considered to be one of the key fibrinolytic modulators on the surface of endothelial cells.7

Considering the hypercoagulable state of most vascular diseases, including atherosclerosis, the possibility of modulation of fibrinolytic activity by annexin II is of great interest. Recently, annexin V, another annexin family member, was shown to be effective in reducing thrombus formation in a rat carotid artery injury model by using a mechanism that may involve inhibition of platelet activity in the injured vessel.10

In this study, we examined the potential role of annexin II in a rat carotid artery model utilizing a recombinant annexin II (rAN II) protein. Our results indicate that intravenous administration of annexin II significantly inhibits thrombus formation in vivo without affecting systemic hemostatic parameters, and may demonstrate its potential as a novel therapeutic tool for vascular diseases.

Materials and Methods

Cell Culture and Reagents

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords and cultured as described previously.11 Antibodies used in this study were as follows: monoclonal murine anti-human annexin II (purchased from Zymed, South San Francisco, Calif), W6/32 (murine anti-human HLA-A, -B, or -C or HLA class I),12 and Penta-His antibody (monoclonal murine anti-histidine antibody, purchased from Qiagen, Hilden, Germany). Lys-plasmin-
oxygen and t-PA were purchased from Technoclone (Vienna, Austria), and a fluorogenic plasmin substrate, d-Val-Leu-Lys-AMC (AMC-081), was obtained from Enzyme Systems Products (Livermore, Calif). BSA was purchased from Wako. PBS was purchased from Moreregate.

**Construction of Recombinant Human Annexin II**

A full-length human annexin II cDNA* was inserted into a histidine-tagged bacterial expression vector, pQE (purchased from Qiagen), between the *KpnI* and *Sal*I sites (pQE-AN II). A mutant AN II (mAN II) expression vector that lacks 224 amino acids of the C-terminal was created as follows. A stop codon was introduced at 114 amino acids using an oligonucleotide-based mutagenesis kit (Quick Change, Clontech) and two complement oligonucleotides 5'-GCTTCTGAGCTATAGGCTTCCATGAAG-3' and 5'-CTTCTAG-GAACTTATAGCTCAGAAG-3' according to the manufacturer's instructions. The mutated sequence was confirmed by DNA sequencing. A cell lysate prepared from overnight cultures of pQE-AN II-transformed JM109 was pelleted (8000 rpm, 15 minutes), resuspended in a buffer containing 50 mmol/L sodium phosphate and 300 mmol/L NaCl (pH8), and sonicated for 2 minutes. The lysate was purified using a nickel-affinity column (His-Trap, purchased from Amersham Pharmacia). The purity of the rAN II protein and mAN II was confirmed by SDS-PAGE followed by silver staining.

**Fluorescent Immunobinding Assay**

HUVECs were plated in a 96-well culture plate and treated with the indicated amount of rAN II, BSA, or medium in the presence of 0.1 mL of RPMI 1640+1% FBS alone for 1 hour at 37°C. A fluorescent immunosassay was then carried out as previously described.1,2 Briefly, HUVEC monolayers were washed two times with 0.1 mL of RPMI 1640+1% FBS and incubated on ice with either anti-human annexin II or W6/32 at the concentration of 10 μg/mL in RPMI 1640+1% FBS for 45 minutes. The plates were then washed twice with RPMI 1640+1% FBS and incubated with FITC-conjugated goat anti-mouse IgG (1:50, purchased from Caltag) diluted 1:50 in Dulbecco's PBS (DPBS) containing 0.9 mmol/L CaCl₂ and 0.33 mmol/L MgCl₂, on ice for 45 minutes. Plates were then washed twice with DPBS+0.5% FBS and twice with DPBS alone. Cells were then lysed with 0.15 mL of 0.01% NaOH in 0.1% SDS, and the fluorescent intensity was quantified using a fluorescent plate reader (Cytofluor II, Perseptive Biosystems).

**Fluorescent Immunohistochemistry**

Monolayer-associated rAN II was also examined by fluorescent immunohistochemical analysis. HUVECs were plated in a 12-well culture plate, and treated with 100 ng/mL of rAN II, mAN II, or BSA in the presence of 0.8 mL of RPMI 1640+1% FBS for 1 hour at 37°C. HUVEC monolayers were washed two times with 0.8 mL of RPMI 1640+1% FBS and incubated at room temperature with anti-histidine antibody at a concentration of 10 μg/mL in RPMI 1640+1% FBS for 45 minutes. Plates were then washed three times with RPMI 1640+1% FBS and incubated with Cy3-conjugated goat anti-mouse IgG (1:50, purchased from Molecular Probes) that was diluted 1:500 in PBS containing 0.9 mmol/L CaCl₂ and 0.33 mmol/L MgCl₂ on ice for 45 minutes. The plates were then washed twice with DPBS+0.5% FBS and twice with DPBS alone. Fluorescent intensity from HUVECs was observed using a fluorescent microscope (IX70, Olympus).

**Fibrinolytic Activity of Recombinant Human Annexin II**

The fibrinolytic activity of annexin II was measured in the presence of Lys-plasminogen and t-PA as described previously.6 The indicated amount of rAN II, mAN II, or BSA protein was added directly into a 96-well culture plate, or on HUVEC monolayers plated in a 96-well culture plate and cultured for 1 hour in the presence of 0.1 mL of RPMI 1640+1% FBS. The plates were then washed two times (100 μL PBS/well) and then exposed to Lys-plasminogen (10 μg/mL in PBS, 37°C, 1 hour). After two additional washes, a substrate mixture consisting of PBS containing t-PA (200 mmol/L) and a fluorogenic plasmin substrate, d-Val-Leu-Lys-AMC (100 mmol/L), were added to each well (100 μL/well), Plasmin generation in each well was measured at 15-minute intervals, with excitation set at 360 nm and emission at 460 nm, using a fluorescent plate reader, and expressed as relative fluorescent units/min² (RFU/min²).

**Homocysteine (Hcy) (100 μmol/L) (purchased from Sigma) and plasminogen activator inhibitor-1 (PAI-1) (150 μg/mL) (purchased from Technoclone) were added to HUVECs in a 96-well culture plate. After 1 hour, the plates were washed twice with PBS. rAN II was then added to the HUVEC monolayers and cultured for 1 hour in the presence of 0.1 mL of RPMI 1640+1% FBS. Plasmin generation was analyzed as described above.

**Rat Carotid Artery Thrombosis Model**

All procedures on animals were approved by the Institutional Animal Care Committee of Tokyo Medical and Dental University (approval No. 0010209) and conducted according to the Guidelines for Animal Experimentation in Tokyo Medical and Dental University. Animals were supplied from Saitama Experimental Animal Supplier (Saitama, Japan). A rat carotid artery thrombus model was constructed as described previously.11 In brief, male Sprague-Dawley rats (body weight 250 g) were anesthetized with intraperitoneal pentobarbital (20 mg/kg). The animals were then injected intravenously with rAN II, mAN II, or BSA (1 mg/kg each). The carotid artery of the rat was exposed and covered with filter paper that had been soaked in 35% ferric chloride (FeCl₃). The patency of the artery was monitored using a Doppler flow probe (type HPD10-20S, Crystal Biotechnology Institute) attached to the carotid artery adjacent to where the filter paper was placed. The Doppler flow probe was analyzed using PowerLab/SSP (AD Instruments Japan). In some experiments, the FeCl₃-subjected area of the carotid artery was excised, fixed with formalin, and embedded in paraffin. To check the coagulation parameters, bleeding time was measured by the Duke method. A 2-mm deep nick was made in the ear lobe to cause bleeding. The test was timed from the start of bleeding until it was completely stopped. To avoid bias, animals were blindly assigned to the examiners. Blood was collected at the end of the experiments in the presence of 3.8% sodium citrate, and the obtained plasma samples were used to measure the prothrombin time (Quick-One-Step method, SRL, Inc) and active partial thromboplastin time (Langdell method, SRL, Inc).

**Histological Analysis**

Five-micrometer sections from formalin-fixed, paraffin-embedded carotid artery specimens were placed onto poly-L-lysine–coated slides; deparaffinized by three changes of xylene; rehydrated in 100%, 90%, and 80% ethyl alcohol; and then stained by hematoxylin and eosin. Microscopic images were captured by a charge-coupled device camera–equipped light microscope (IX70, Olympus) and fed into a computer, after which intimal patency was calculated using image analysis software (IP Laboratory). The patency ratio was calculated from five serial cross sections in each condition as follows: patency ratio (%) = (L - T/L) × 100, where L = area of artery lumen within the intimal lining (mm²) and T = area covered with thrombus (mm²).

**Fluorescent Immunohistochemical Analysis**

Sections of the rat carotid artery segments were prepared as described above and incubated with anti-histidine monoclonal antibody (mAb) diluted 1:500 in PBS for 1 hour. Specimens were washed three times with PBS with 0.5% Tween-20 and incubated with Cy3-conjugated goat anti-mouse IgG diluted 1:100 in PBS for 1 hour. The specimens were then washed three times with PBS with 0.5% Tween-20. Fluorescence was observed using fluorescent microscopy (IX70, Olympus).

**Western Blotting**

Artery segments were minced and homogenized in a Dounce homogenizer in the presence of 250 μL of lysis buffer (containing, in mmol/L, benzamidine 20, PMSF 1, and Na₂VO₃ 1; 10 μg/mL pepstatin A; 1 μg/mL leupeptin; and 0.1% Triton X-100) and
incubated on ice for 2 minutes. The lysates were centrifuged at 15,000 rpm for 15 minutes and removing sample buffer (3×) was added to the supernatant. Aliquots (10 μg) of the samples were subjected to 12.5% SDS-PAGE, transferred to a polyvinylidene fluoride membrane, and incubated with 2.5% BSA in TBS-T (20 mmol/L Tris, 137 mmol/L NaCl, 0.1% Tween-20, pH 7.6) at 4°C for 16 hours. The membrane was then incubated with Penta-His antibody (10 μg/mL in 2.5% BSA in TBS-T) for 1 hour, washed three times with TBS-T, and incubated with an HRP-conjugated goat anti-mouse IgG for 1 hour. After washing again three times with TBS-T, the protein was detected using an enhanced chemiluminescence kit (purchased from Amersham Pharmacia Biotech).

Statistical Analysis

Data are presented as mean ± SD as indicated. Two-tailed Student t tests were performed with Microsoft Excel. Probability values represent the results of these t tests, and values of \( P < 0.05 \) were considered statistically significant.

Results

Preparation of rAN II Protein

rAN II and its mutant form (mAN II) were produced and purified as described in Materials and Methods. mAN II lacks the C-terminal 224 amino acids important in phospholipid binding to plasminogen. We confirmed the purity and abundance of these proteins by SDS-PAGE followed by silver staining (data not shown).

To analyze fibrinolytic activity, the plasmin-generating abilities of solid-phase rAN II and mAN II, after coating on a plastic plate, were compared with those of the control protein (BSA). As shown in Figure 1A, rAN II contributed to significantly more plasmin generation than either mAN II or BSA (no protein, 0.42±0.06 RFU/min\(^2\); rAN II, 5.34±0.23 RFU/min\(^2\) \( P < 0.01 \); mAN II, 1.44±0.26 RFU/min\(^2\); BSA, 0.88±0.16 RFU/min\(^2\) [mean±SD, \( n = 5 \)]; protein concentration, 50 ng/mL).

To examine the fibrinolytic activity of rAN II under more physiological conditions, we measured the plasmin generation of HUVECs pretreated with rAN II, mAN II, or BSA. Observation with a phase-contrast microscope validated the confluence of the HUVEC monolayer in each well (data not shown). The presence of rAN II on the surface of HUVECs was confirmed by a fluorescent immunoassay using anti-annexin II mAb before measuring plasmin generation (Figure 1B). We verified rAN II as well as the absence of mAN II and BSA, on the surface of cultured vascular endothelium using fluorescent microscopy (Figure 1B). Further, positive staining of constitutively expressed HLA class I (W6/32) validated the presence of HUVECs in each well (data not shown). rAN II significantly enhanced plasmin generation in HUVECs, as compared with mAN II or BSA (no protein, 1.87±0.33 RFU/min\(^2\); rAN II, 3.45±0.15 RFU/min\(^2\) \( P < 0.01 \); mAN II, 2.40±0.14 RFU/min\(^2\); BSA, 1.92±0.27 RFU/min\(^2\) [\( n = 5 \)]; protein concentration, 50 ng/mL) (Figure 1C). rAN II–dependent plasmin generation was also higher in blank wells than in wells in which cells were cultured. This may have been due to preferential binding of rAN II to a plastic surface rather than to the surface of endothelial cells; thus, a detailed characterization of rAN II binding should be further performed. In our experiments with HUVECs, the integrity of the monolayers was checked, and the binding of rAN II to the plastic surface could be ignored.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** A, Plasmin generation of solid-phase rAN II. Indicated concentrations of rAN II (solid bars), mAN II (shaded bars), or BSA (open bars) were added to a plastic plate and cultured for 1 hour in the presence of 0.1 mL of RPMI 1640+1% FBS. Plasmin generation was analyzed by measuring fluorescent activity from the fluorogenic plasmin substrate and compared with wells without treatment, as described in Materials and Methods (\( n = 5 \)). Data are representative of three independent experiments. \( P < 0.01 \) vs mAN II or BSA. B, Fluorescent immunoassay of HUVECs treated with rAN II, mAN II, or BSA. HUVECs were plated in a 12-well culture plate and treated with rAN II, mAN II, or BSA at a concentration of 100 ng/mL. HUVECs were then incubated with an anti-histidine mAb followed by Cy3-labeled anti-mouse IgG as described in Materials and Methods. Fluorescent intensity from the HUVECs was observed with a fluorescent microscope (IX70, Olympus) (magnification ×400; scale bar = 10 μm). Fluorescent immunoassays of cell surface annexin II in unactivated HUVEC monolayers treated with rAN II, mAN II, or BSA at 10, 50, and 100 ng/mL were performed using an mAb against annexin II, as described in Materials and Methods (\( n = 4 \)). Data shown are representative of three independent experiments. \( P < 0.01 \) vs mAN II– or BSA-treated group. C, Plasmin generation of rAN II on surface of HUVECs. HUVEC monolayers were incubated in the absence (–) or presence of 50 ng/mL of rAN II, mAN II, or BSA for 1 hour. Plasmin generation was analyzed as described above (\( n = 5 \)). Data shown are representative of three independent experiments. \( P < 0.01 \) vs control or mAN II– or BSA-treated group.

rAN II Restored Impaired Fibrinolytic Activity Induced by Hcy and PAI-1

Both Hcy and PAI-1 are considered to disrupt the fibrinolytic process on the surface of endothelium.\(^3\) Hcy has been reported to be a coronary risk factor\(^14\) and was recently reported to decrease fibrinolytic activity by inhibiting the
binding of t-PA to cell surface annexin II. When HUVECs were incubated in the presence of Hcy (100 μmol/L) and PAI-1 (150 mU/mL), plasmin generation was significantly diminished (Hcy, 52.31 ± 12.30% of control [P < 0.01]; PAI-1, 31.71 ± 14.63% of control [P < 0.01, n = 5]); however, it was restored by the addition of rAN II (Hcy, 152.27 ± 12.25% of control; PAI-1, 77.85 ± 5.00% of control [n = 5]), but not mAN II (Hcy, 38.21 ± 18.66% of control [n = 5]) or BSA (Hcy, 21.29 ± 23.02% of control; PAI-1, 37.40 ± 11.34% of control [n = 5]) (Figure 2).

**rAN II Reduced Thrombus Formation in Rat Carotid Artery Thrombus Model**

On the basis of these in vitro data, we then evaluated the effect of rAN II in a rat carotid artery thrombus model in vivo. Male Sprague-Dawley rats were intravenously injected with rAN II, mAN II, or BSA (1 mg/kg each) via the tail vein. The carotid artery was then exposed and covered with 35% FeCl₃-infiltrated filter paper to induce thrombus formation in a manner previously reported. To measure the amount of blood flow in the carotid artery, a Doppler flow probe was applied to the injured artery adjacent distal to the thrombosed area. There was a time-dependent reduction of blood flow seen in rats treated with control BSA, suggesting the formation of occlusive thrombus in these areas. The blood flow was completely shut off within 14.76 ± 3.63 minutes (n = 7) in BSA-treated rats. In contrast, rats treated with rAN II exhibited significantly less reduction of blood flow (37.13 ± 14.61 minutes [n = 7]). Those treated with mAN II exhibited a reduction of blood flow similar to that observed in the controls (17.23 ± 7.21 minutes [n = 7]) (Figure 3A). Typical patterns of blood flow during these experiments are illustrated in Figure 3B. In the case of mAN II– and BSA-treated animals, carotid flow quickly decreased, whereas in the case of rAN II–treated animals, carotid flow was sustained for >15 minutes. Further, a sharp recovery of flow (indicated by an arrow in the Figure) was observed in rAN II– and mAN II–treated rats, however, not in those treated with BSA. To validate the formation and reduction of thrombus, sections of the carotid artery specimen prepared from FeCl₃-treated carotid arteries 10 minutes after thrombus induction were stained with hematoxylin-eosin and examined by a light microscope. At this time point, the patency ratio of the vessel lumen was 27.99 ± 13.52% (n = 8) in the BSA-injected group.
and enhanced thrombin generation. Therefore, modulation of thrombogenesis and fibrinolysis has been a major focus in atherosclerosis research.

Annexin II, a member of the annexin family, is a 36 000-Da calcium-regulated, phospholipid-binding protein. It has been reported as a coreceptor for both plasminogen and t-PA. In this study, we constructed a recombinant annexin II protein (rAN II) and measured its enhancement of fibrinolytic activity. rAN II, but not its mutant form (mAN II), contributed to plasmin generation on a plastic plate (Figure 1A) as well as on a confluent monolayer of HUVECs (Figures 1B and 1C). To further understand the role of rAN II in more pathophysiological conditions, we examined its effect on vascular endothelium treated with the procoagulant reagents, Hcy and PAI-1, which may play a critical role in the atherogenic process via dysregulation of endothelial thromboresistance. Previous reports indicate that Hcy inhibits annexin II–dependent cell surface fibrinolytic activity. PAI-1, an endogenous inhibitor of plasmin generation, has also been shown to contribute to thrombus formation. In genetically engineered PAI-1 knockout mice, thrombus formation 24 hours after vascular injury was significantly reduced when compared with that observed in wild-type animals.

As shown in Figure 2, treatment of endothelial cells with either PAI-1 or Hcy significantly decreased plasmin generation. However, when coincubated with rAN II, the impaired plasmin generation was almost completely restored. These data indicated that rAN II has a capability of restoring impaired fibrinolytic activity on the endothelial cell surface. Although the mechanisms involved in the pathogenicity of Hcy and PAI-1 have not been fully elucidated, our data support a mechanism involving impaired plasmin generation. In our study, these compounds inhibited endothelial cell surface plasmin generation and excess annexin II appeared to restore fibrinolytic activity.

To more critically assess the fibrinolytic activity of rAN II in vivo, we then used an FeCl3-induced rat carotid artery thrombosis model. FeCl3-induced thrombi have been shown to be similar in cellular composition and fibrin content to those formed in human coronary arteries. These thrombi consist of numerous red blood cells and platelets with a fibrin meshwork, and the degree of formation was found to be dependent on the concentration and exposure duration of FeCl3, applied to the carotid artery. We chose 35% FeCl3 because this concentration reproducibly caused thrombotic occlusion of the vessel in rats weighing 250 to 300 g (data not shown). Continuous measurement of carotid blood flow using

**Discussion**

Arterial thrombus formation has been implicated in most episodes of acute myocardial infarction or in brain infarction. The procoagulant status of the vessel wall due to vascular injury or atherosclerosis leads to adhesion and accumulation of platelets, initiation of coagulation cascades,
a Doppler module clearly documented that administration of rAN II, but not BSA or mAN II, contributed to blood flow in the injured arteries. Histological examination (Figures 4a through 4c) confirmed the presence (in the case of BSA or mAN II) or absence (in the case of rAN II) of thrombus in carotid arteries subjected to FeCl₃. mAN II lacks 224 amino acids of the C terminus, which contains a plasminogen binding site (307th amino acid lysine). These data suggest the importance of the C-terminal plasminogen binding site for the fibrinolytic activity of annexin II. Furthermore, mAN II lacks three out of four calcium-dependent phospholipid binding motifs that mediate the binding of annexin II to the plasma membrane. This weak binding to the plasma membrane may also account for the lack of fibrinolytic activity of mAN II. Of note, the flow pattern of mAN II–treated carotid arteries was similar to that of rAN II, although they occluded much faster than those treated with rAN II. These results suggest that mAN II may also alter the thrombosis process in the rat carotid arteries. Although the mechanism by which rAN II prevents FeCl₃–induced thrombus formation has yet to be elucidated, our in vitro data suggest that enhancement of local plasmin generation within FeCl₃–treated carotid arteries may counteract local fibrin formation. The relatively unchanged level of systemic coagulation parameters was also interesting when we considered the relatively high amounts of recombinant proteins (300 μg) used. One explanation may be that because annexin II protein has a high affinity for phospholipid, the majority of exogenous annexin II may bind to plasma membranes soon after injection rather than remaining free in the circulating plasma. We would like to further investigate the systemic and local effects of intravenous annexin II injection using experimental animal models, to establish a potential therapeutic approach for thrombogenic vascular disease.

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