Antithrombin III-β Associates More Readily Than Antithrombin III-α With Uninjured and De-endothelialized Aortic Wall In Vitro and In Vivo

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The properties of two isoforms, α and β, of rabbit antithrombin III (ATIII) were compared in the presence of undamaged or de-endothelialized rabbit aortic wall. Similar quantities of ATIII-α and ATIII-β bound to and rapidly saturated the endothelium in vitro, but the rate of transendothelial passage of ATIII-β exceeded that of ATIII-α by 22%. Furthermore, ATIII-β was adsorbed approximately twice as rapidly as ATIII-α by the subendothelium of the de-endothelialized aorta. Binding of both isoforms was decreased (ATIII-β more than ATIII-α) by pretreating the subendothelial surface with heparitinase. Also, subendothelium-bound ATIII-β was desorbed more readily than bound ATIII-α by thrombin. In vivo, the rate of uptake of iodine-131-labeled ATIII-β from the circulation by the aortic wall and the major organs was 30–50% faster than that of iodine-125-labeled ATIII-α. In contrast, the uptake of 125I-ATIII-β by the de-endothelialized aorta in vivo was three times faster than that of 131I-ATIII-α. By these criteria, ATIII-β is the more active of the two isoforms. We surmise that plasma and, consequently, vessel wall levels of ATIII-β may be vital for controlling thrombogenic events caused by injury to the vascular wall. (Arteriosclerosis and Thrombosis 1991;11:530–539)

From recent reports, two forms of the glycoprotein antithrombin III (ATIII) have been isolated from rabbit and human plasma. Both the rabbit and human isoforms were separated by gradient elution from a heparin-Sepharose affinity column, the major human isoform, ATIII-α, eluting at 0.9–1.0 M NaCl, and the minor isoform, ATIII-β, at 1.4 M NaCl. Human ATIII-β was shown to contain approximately 25–30% less carbohydrate than ATIII-α. More recently, Brennan et al. have reported that, unlike the human ATIII-α isoform, which has four biantennary N-glycans (at asparagine [Asn] 96, 135, 155, and 172), the ATIII-β isoform possesses only three N-glycans, lacking that at Asn 135. Carrell et al. have suggested that the absence of the N-glycan at Asn 135 may extend the binding site for heparin and, consequently, increase the affinity of ATIII-β for heparin.

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Previous studies from this laboratory have attempted to understand the behavior of rabbit ATIII at the aortic wall in vitro and in vivo. From the report of Carlson and Atencio, we have concluded that our investigations to date have been made only with the major isoform from rabbit plasma (referred to as ATIII-α in Reference 1). This form of ATIII was bound, largely irreversibly, by the aortic endothelium in vitro, and its passage across the endothelium caused the protein to accumulate in the subendothelial space. Pretreatment of the aorta with glycosaminoglycan-degrading enzymes did not inhibit binding of ATIII to the endothelium. However, pretreatment of the aortic subendothelium with either heparitinase or thrombin, but not plasmin, rapidly decreased ATIII binding and also selectively removed the heparan sulfate proteoglycan from the basement membrane of medial smooth muscle cells. From these data, we concluded that extracellular heparan sulfate chains are responsible for binding ATIII in the arterial subendothelium.

In the following account, we have compared the properties of the two isoforms of rabbit ATIII, named α and β, towards the rabbit aortic wall in vitro and in vivo. ATIII-α and ATIII-β in the following report are equivalent, respectively, to ATIII-α and ATIII-vh assigned to the rabbit ATIII isoforms from their relative affinities to heparin-Sepharose.
Materials

Rabbit plasma

Methods

Isolation of Antithrombin III Isoforms From Rabbit Plasma

The procedure of Carlson and Atencio was used, with a few modifications. Rabbit acid-citrate-dextrose (ACD) plasma (200 ml) was fractionated by adding solid (NH₄)₂SO₄ to 40% saturation, and the dialyzed supernatant (=260 ml) was chromatographed on heparin-Sepharose (9x2.2 cm; prepared from Pharmacia Ltd., Dorval, Canada; S-2238 from Kabivitrum, Stockholm, Sweden; Eagle’s minimum essential medium (MEM) from GIBCO, Burlington, Canada; Thrombostat from Parke-Davis Co., Detroit, Mich.; Enzymobeads and materials for polyacrylamide gel electrophoresis (PAGE) and immunoblotting from Bio-Rad Ltd., Mississauga, Canada; alkaline phosphatase–linked antibodies to hen immunoglobulin G (IgG) and rabbit IgG from Zymed Labs Inc., San Francisco, Calif., and Bio-Rad, respectively; Immobilon from Millipore, Bedford, Mass.; and [³¹I]NaI from Du Pont–NEN, Wilmington, Del.

Purity and Properties of Antithrombin III Isoforms

Purity was assessed by PAGE with 7.5% gels in the presence of 0.1% sodium dodecyl sulfate (SDS). After electrophoresis, the protein in the gel was either fixed and stained with 0.1% Coomassie Brilliant Blue or transferred to Immobilon for immunologic identification by the method recommended by Millipore. For this purpose, a polyclonal antibody to rabbit ATIII was raised in a laying hen, and IgG was extracted from the egg yolks. After development, the blots were scanned with a laser densitometer (LKB Ultrascan, Bromma, Sweden).

Radiolabeling of ATIII isoforms (α with iodine-125, β with iodine-131) was undertaken by the immobilized lactoperoxidase–glucose oxidase (Enzymobead) procedure as described by the manufacturer (Bio-Rad). Radioactivity was measured with a Beckman Model 5500 gamma counter (Beckman Instruments, Palo Alto, Calif.). Approximately 10⁶ dpm/μg protein was obtained by this method. Preparations of the radiolabeled isoforms were subjected to SDS-PAGE for comparison with unlabeled ATIII, and the gel, after it was stained and dried, was exposed to Kodak X-AR5 film.

For measurement of antithrombin activity, bovine α-thrombin was isolated and purified from Thrombostat by a procedure derived from the method of Lundblad et al. After SDS-PAGE, purified thrombin appeared as a major band (36 kd) and a minor band (28 kd). A polyclonal antibody against bovine FPR-thrombin (i.e., thrombin inactivated by D-PhePro-L-Arg-CH₂Cl) was raised in rabbits and was used to identify thrombin after blotting. Using this method, the 36-kd and 28-kd bands stained positively and were assumed to be α- and β-thrombin, respectively. Specific coagulant activities of purified enzyme preparations ranged from 2,000 to 2,200 IU/mg using a plasma (human) coagulation assay. Antithrombin activity was measured by incubating individual isoforms (284 pmol) with thrombin (28.4 pmol) in 0.05 M Tris HCl–0.15 M NaCl, pH 7.35, at 23°C for as long as 60 minutes. In some experiments, heparin (0.284 pmol) or heparan sulfate (2.84 pmol) was added; molar values were calculated by using 15,000 and 20,000 for heparin and heparan sulfate, respectively. Residual thrombin was measured by taking an aliquot of the incubate for reaction with S-2238 and was calculated relative to a standard thrombin-response curve obtained under similar conditions.

Excision and Preparation of Thoracic Aortas From Rabbits

New Zealand White rabbits (2.5–3.2 kg) were anesthetized (35 mg sodium pentobarbital/kg body wt) and exsanguinated by cannulation of either carotid artery. After exsanguination, a de-endothelializing injury to the rinsed aorta was performed in situ in some rabbits by passing a Fogarty balloon catheter (type 12-040-4F, Edwards Laboratories Inc., Santa Ana, Calif.), filled with 0.5 ml saline, twice through the lumen of the vessel when the latter was immersed in MEM containing RSA (0.4%, wt/vol). The aorta was excised, rinsed, and prepared as seven 1-cm-long segments for experiment as described previously.

Interaction Between Antithrombin III Isoforms and Rabbit Aortic Wall In Vitro

All incubations were conducted at 37°C. Samples of ¹²⁵I–ATIII-α and ¹³¹I–ATIII-β were incubated with aortic segments in 1 ml 4% RSA-MEM for various times. After incubation, each vessel segment was rinsed (4 ml MEM, 1 minute), the surface area was...
measured, and the vessel layers (endothelium, intima-media, and adventitia) were separated and measured for radioactivity content as described previously.\textsuperscript{18} Accessibility of aorta-bound ATIII was measured at 3 and 12 hours after injecting various concentrations of heparitinase (0.25-1.9 units/ml 4% RSA-MEM) was performed for 10 minutes before rinsing (4 ml MEM, 1 minute). The pretreated vessel segment was then incubated (10 minutes) with the radiolabeled ATIII isoforms.

**Behavior of Antithrombin III Isoforms In Vivo**

The organ distribution of ATIII-\(\alpha\) and ATIII-\(\beta\) was measured at 3 and 12 hours after injecting rabbits with the radiolabeled isoforms (120-160 pmol each/kg). Blood samples (\(\sim 0.85\) ml blood into 0.15 ml ACD\textsuperscript{20}) were taken 5 minutes after injection and during exsanguination. After exsanguination, each excised organ was rinsed, weighed (after first removing fat and connective tissue), and homogenized (1.5 M NaCl containing 2-octanol), much as described previously.\textsuperscript{21} Aliquots (in triplicate) of a known volume of each organ homogenate and plasma samples were measured for radioactivity content. The specific radioactivity of each organ (i.e., counts per minute per gram of tissue) was compared with the radioactivity of 1 ml blood at exsanguination.

Adsorption of the radiolabeled isoforms by the aortic wall was determined for the undamaged vessel and for the aorta after balloon de-endothelialization. A de-endothelializing injury to the aorta in vivo was inflicted by passing a balloon catheter through a femoral artery as previously described,\textsuperscript{18} which caused complete denudation of the aortic surface. The effect of such an injury on plasma levels of ATIII-\(\alpha\) and ATIII-\(\beta\) was determined by inflicting the balloon injury 10 minutes after injection and allowing the radiolabeled isoforms to circulate for as long as 3 hours. The aorta was recovered from the exsanguinated rabbit and cut into seven 1-cm-long segments. Radioactivity measurements were made on the separated vessel layers (for undamaged aortas, endothelium, intima-media, and adventitia; for balloon de-endothelialized aortas, platelet monolayer, intima-media, and adventitia) as explained previously.\textsuperscript{8} The radioactivity bound by the aortic surface (i.e., counts per minute per square centimeter) was related to the quantity of radioactivity in the circulation (i.e., counts per minute per milliliter of blood) at the time of euthanization.

**Results**

**Isolation and Characterization of Antithrombin III Isoforms**

Gradient elution of ATIII from heparin–Sepharose separated ATIII-\(\alpha\) (displaced at 0.8 M NaCl) from ATIII-\(\beta\) (1.4 M NaCl). From five preparations, the recovery of ATIII-\(\alpha\) and ATIII-\(\beta\) amounted to 14.55±2.07 mg (mean±SD) and 1.43±0.15 mg/100 ml plasma, respectively. Six analyses revealed that ATIII-\(\alpha\) (8.11±0.53 residues/mol) contained two more sialic acid residues than did ATIII-\(\beta\) (6.03±0.45 residues/mol).

The results of SDS-PAGE of the purified isoforms, before and after radiolabeling, are shown in Figures 1a and 1b, respectively. By an immunoblotting procedure, the molecular size of ATIII-\(\alpha\) (\(M_r\), 63 kd) was found to be significantly greater than that of ATIII-\(\beta\) (\(M_r\), 61 kd), and both isoforms were present in rabbit plasma and in detergent extracts of intima-media from undamaged and de-endothelialized rabbit aortas (Figure 1a). Extracts from undamaged aortas (Figure 1a; lanes 4 and 5) appeared to contain more ATIII-\(\beta\) than ATIII-\(\alpha\), although the presence of high-molecular-weight ATIII-positive bands (complexes?) were the dominant components. Extracts from aortas that had been de-endothelialized in vivo contained a greater proportion of the high-molecular-weight components (lanes 2 and 3).

The radiolabeled isoforms were found to be free of visible contaminants as shown by SDS-PAGE–autoradiography (Figure 1b). Reaction with thrombin produced stable thrombin–ATIII complexes for both radiolabeled isoforms.

**Inactivation of Thrombin by Antithrombin III Isoforms**

Reaction progress was followed by enzyme assay (Figure 2) and by SDS-PAGE (Figure 3).

In the absence of heparin, thrombin was inactivated slowly by ATIII-\(\alpha\) or ATIII-\(\beta\); rate constants were calculated to be 1.67×10\textsuperscript{3} sec\textsuperscript{-1} and 2.17×10\textsuperscript{3} sec\textsuperscript{-1}, respectively. However, the addition of heparin in catalytic amounts (i.e., molar ratio of ATIII to heparin=1,000) increased the rate of inactivation by both isoforms; of ATIII-\(\alpha\) to 8.03×10\textsuperscript{3} sec\textsuperscript{-1} and of ATIII-\(\beta\) to 11.67×10\textsuperscript{3} sec\textsuperscript{-1} (Figure 2). A similar effect was observed when heparin was replaced by heparan sulfate, although considerably more heparan sulfate (molar ratio of ATIII to heparan sulfate=100) was required to enhance the rates of inactivation significantly (Figure 2).

Analysis of the thrombin–ATIII complexes by SDS-PAGE showed that the \(\alpha\) and \(\beta\) isoforms reacted with thrombin to yield a similar range of complexes but of slightly staggered position after electrophoresis; the ATIII-\(\beta\) products ran incrementally faster than those corresponding to ATIII-\(\alpha\) (Figures 3a and 3b). Initially, two product bands, thrombin–ATIII complex (99 kd) and the postcomplex type of ATIII (69 kd), were formed during reaction of each isoform with thrombin. Immunoblotting with polyclonal antibody preparations to either rabbit ATIII or to bovine thrombin showed that the 99-kd band contained both ATIII and thrombin. The 69-kd product reacted positively to antibody to ATIII but negatively to antibody to thrombin. Later during
the reaction, a third product (93 kd) appeared, increasing in intensity for as long as 30 minutes; this band, from immunoblotting, also contained ATIII and thrombin. Last, the band of a fourth product (82 kd) was observed, which contained ATIII and thrombin. These latter two complexes (93 kd and 82 kd) were particularly prominent when the reaction between thrombin and ATIII was performed in the presence of excess thrombin (data not shown).

Interaction of Antithrombin III Isoforms With Aortic Wall In Vitro

Exposing the freshly excised aortic wall to an equimolar mixture of radiolabeled subforms resulted in similar quantities of the α and β isoforms saturating the endothelium (Figure 4). However, the passage of ATIII-β across the endothelium was marginally but significantly ($p<0.05$) faster than that of ATIII-α by 22%, as shown by comparison of the mean quantities of each radiolabeled isoform bound to the subendothelium during 5, 10, 20, and 30 minutes’ incubation.

Similar experiments were made with rabbit aortas that had been de-endothelialized in vitro. Uptake of ATIII-β by the de-endothelialized aorta was approximately twofold greater than that of ATIII-α, although saturation of the intima-media by either isoform was not clearly observed for incubation times even as long as 30 minutes (Figure 4).

Pretreating the de-endothelialized aorta with heparitinase caused a rapid loss of ATIII binding potential by the subendothelium (Figure 5), but more binding sites for the β isoform (up to 85% destroyed) were lost than for the α form (55%).
To compare the effect of thrombin on each subendothelial-bound isoform, de-endothelialized aortic segments were incubated with $^{125}$I-ATIII-$\alpha$ and $^{131}$I-ATIII-$\beta$ to allow similar quantities of each isoform to be bound to the subendothelium. These segments were then exposed to thrombin (0.75–2.9 IU/ml). Some desorption of both isoforms (presumably as thrombin–ATIII complexes) from the vessel wall was observed relative to the subendothelium-bound isoforms incubated in 4% RSA-MEM alone (Figure 6). At a thrombin concentration of 0.75 IU/ml, the $\beta$ isoform was displaced six times more readily than $\alpha$.

**Behavior of Antithrombin III Isoforms In Vivo**

The relative distributions of the two isoforms in the major organs were measured 3 and 12 hours after intravenous injection (Table 1). At 3 hours, the $\beta$ form was present, as radioactivity, in greater quantity (28–67%) than the $\alpha$ form in all organs studied; at 12 hours, the relative accumulation of the $\beta$ isoform had increased in some organs (ileum, lung, aorta, and kidney) but had decreased in the liver and spleen.

Uptake of the isoforms by the endothelium and subendothelium of the undamaged aorta 3 hours after injection is compared in Figure 7a. Adsorption of radiolabeled ATIII-$\alpha$ by the endothelium was greater than that for ATIII-$\beta$. However, relative to circulating levels of the radiolabeled isoforms, the $\beta$ isoform was preferentially transported across the endothelium into the subendothelium (0.10 pmol/cm²/hr) compared with $\alpha$ (0.76 pmol/cm²/hr) during the first hour after injection. Saturation levels of the subendothelium 3 hours after injection amounted to 1.34 pmol/cm² and 0.19 pmol/cm² for ATIII-$\alpha$ and ATIII-$\beta$, respectively (Figure 7a).
The effect of de-endothelialization in vivo increased the rate of uptake of both radiolabeled isoforms by the aortic wall substantially (Figure 7b). The β isoform was adsorbed three times faster than α, and saturation of the subendothelium by both isoforms was observed approximately 30 minutes after balloon injury; at saturation, the quantity of subendothelium-bound α and β isoforms (including platelet monolayer) amounted to 4.92 and 1.29 pmol/cm² of intima–media, respectively. The fraction (expressed as a percentage bound per

**Figure 5.** Line plot of effect of heparitinase (IU/ml) pretreatment of de-endothelialized aortic surface on the binding of radiolabeled isoforms (α, β) of antithrombin III (AT-III, fmol/cm²). Binding conditions (see text) were chosen to obtain approximately equal concentrations of each isoform bound by subendothelium (intima–media). Results are recorded as mean±SD of four segments.

<table>
<thead>
<tr>
<th>ATIII-α</th>
<th>ATIII-β</th>
<th>Ratio β:α</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gut</td>
<td>13.9±3.8</td>
<td>1.78±4.3</td>
</tr>
<tr>
<td>Spleen</td>
<td>18.5±3.8</td>
<td>29.3±4.9</td>
</tr>
<tr>
<td>Liver</td>
<td>24.7±11.0</td>
<td>36.9±16.0</td>
</tr>
<tr>
<td>Lung</td>
<td>32.0±4.4</td>
<td>45.1±10.8</td>
</tr>
<tr>
<td>Kidney</td>
<td>64.2±21.6</td>
<td>107.4±14.7</td>
</tr>
<tr>
<td>Aorta*</td>
<td>0.095±0.016</td>
<td>0.137±0.016</td>
</tr>
</tbody>
</table>

**Figure 6.** Line plot showing comparison of reactivity of subendothelium-bound isoforms (α, β) of antithrombin III (AT-III) towards thrombin (IU/ml). Approximately equal concentrations of labeled isoforms were bound (fmol/cm²), during 10-minute incubation, by subendothelium of de-endothelialized aortic segments. After rinsing (1 minute), segments were exposed to various concentrations of thrombin for 10 minutes. Residual bound radioactivity was assumed to represent the unreacted portion of each isoform that remained after treatment with thrombin or 4% rabbit serum albumin–Eagle’s minimal essential medium. Results are given as mean±SD for four aortic segments.

**Table 1.** Organ Distribution of Radiolabeled Antithrombin III Isoforms in Three Rabbits at 3 and 12 Hours After Injection

<table>
<thead>
<tr>
<th>Organ</th>
<th>3 hours</th>
<th>12 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Distribution (%/g⁻¹ml⁻¹·blood)</td>
<td>ATIII-α</td>
</tr>
<tr>
<td>Gut</td>
<td>13.9±3.8</td>
<td>17.8±4.3</td>
</tr>
<tr>
<td>Spleen</td>
<td>18.5±3.8</td>
<td>29.3±4.9</td>
</tr>
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<td>Aorta*</td>
<td>0.095±0.016</td>
<td>0.137±0.016</td>
</tr>
</tbody>
</table>

Results are expressed as percentages of radioactivity associated per gram of organ relative to that in 1 ml blood at exsanguination. ATIII, antithrombin III.

*For the aorta (intima–media layer), results are percentages of radioactivity per square centimeter per milliliter blood at exsanguination.
milliliter of radioactivity in blood) of α and β that associated with the platelet monolayer covering the denuded aorta at various times after de-endothelialization is given in Table 2. The ratio, β/α, which was reasonably constant over 3.5 hours, revealed that of the two radiolabeled isoforms, approximately 40% more β than α was associated with thrombin-dependent platelet adhesion and aggregation at the damaged vessel wall. However, with respect to the total ATIII adsorbed by the intima–media of the damaged vessel (Figure 7b), the quantity of each isoform associated with the platelet monolayer was small (<10%). Thus, most (>90%) of the ATIII adsorbed by the aorta after de-endothelialization in vivo was associated with the subendothelial tissue rather than with the platelet monolayer.

**Discussion**

The isoforms of rabbit ATIII were qualitatively similar in several of their properties, but overall, the

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>ATIII-α (%)</th>
<th>ATIII-β (%)</th>
<th>Ratio β:α</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>0.026±0.002</td>
<td>0.036±0.007</td>
<td>1.38</td>
</tr>
<tr>
<td>20</td>
<td>0.038±0.003</td>
<td>0.052±0.017</td>
<td>1.37</td>
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<tr>
<td>29</td>
<td>0.050±0.008</td>
<td>0.084±0.020</td>
<td>1.68</td>
</tr>
<tr>
<td>40</td>
<td>0.052±0.017</td>
<td>0.069±0.034</td>
<td>1.33</td>
</tr>
<tr>
<td>85</td>
<td>0.070±0.012</td>
<td>0.093±0.026</td>
<td>1.33</td>
</tr>
<tr>
<td>205</td>
<td>0.093±0.017</td>
<td>0.134±0.013</td>
<td>1.44</td>
</tr>
</tbody>
</table>

ATIII, antithrombin III.

At various times after balloon injury, each rabbit was exsanguinated, and the thoracic aorta was excised. Platelets adsorbed to the vessel luminal surface were separated from subendothelium by cellulose acetate paper. Radioactivity associated with the platelet layer was calculated as a percentage of that in 1 ml blood at exsanguination.
isoform gave a better performance as a thrombin inhibitor than did α. Thus, compared with α, the β isoform inactivated thrombin faster in the presence of either heparin or heparan sulfate (Figure 2). β was adsorbed preferentially by the aortic subendothelium (Figures 4, 7a, and 7b), and, when bound to the subendothelium, β was displaced more readily by thrombin, presumably as a thrombin–ATIII complex (Figure 6). Why rabbit ATIII-β excels is not yet known but, presumably, it is due to a structural alteration in the protein that favors a greater affinity to heparin and to heparan sulfate compared with α.

As shown by SDS-PAGE (Figure 1), rabbit ATIII-β had a slightly decreased molecular weight compared with that of ATIII-α. By comparison, human ATIII-β differs from human ATIII-α by one biantennary N-glycan,3 a difference that has been proposed to account for their characteristic affinities for heparin.3,4 From the analyses of sialic acid content, the rabbit isoforms may also differ by one biantennary N-glycan; that is, eight sialyl residues (equivalent to four biantennary glycans per molecule) are associated with the α isoform and six sialyl residues (three biantennary glycans) with β. However, whether human ATIII-β possesses a greater affinity than ATIII-α for the damaged arterial wall in vivo is not known.

One striking contrast between rabbit ATIII-α and ATIII-β is the ratio of their contents in plasma (ratio of α/β approximately 10) compared with SDS extracts of aortic intima–media (=0.5–1) as measured by densitometry of immunoblots (Figure 1; densitometer results not shown). At present, the site of synthesis of ATIII-β in the rabbit is not known although the major source of ATIII in rat plasma is the liver.23–25 Conceivably, synthesis of both ATIII isoforms by the liver alone could account for the observed levels of ATIII-α and ATIII-β in plasma and in the vessel wall. However, the association of ATIII-α with the vessel wall does not preclude its conversion to ATIII-β within the subendothelium. Such a transformation, using endoglycosidase F in vitro, has been reported for human ATIII-α to ATIII-β,3 but a similar alteration in glycosylation profile (i.e., cleavage of an entire N-glycan at the β-aspartamido linkage) of a plasma glycoprotein in vivo has not been reported. More information on the site(s) and the kinetics of synthesis of the isoforms is needed to understand more fully the respective physiological roles of the two isoforms, particularly after a prothrombotic challenge, for example, damage to the vascular endothelium.

Using the values calculated for ATIII in plasma and the data in Figure 7a, we calculated that the content of the isoforms in the intima–media of the undamaged aorta in vivo amounted to 1.34 pmol/cm² and 0.19 pmol/cm² for α and β, respectively. SDS-extracted ATIII appeared to consist largely of high-molecular-weight components (presumably due to inactive complexes; Figure 1, lanes 4 and 5), and probably only a minor proportion of ATIII contained within the subendothelium of the excised aorta was active bound inhibitor.19 In comparison, a de-endothelializing injury caused greater quantities of both isoforms to associate with the subendothelium during the 30-minute period after injury (Figure 7b), and the subendothelium contained a relatively increased proportion of high-molecular-weight complexes (Figure 1, lanes 2 and 3) compared with the uninjured vessel (lanes 4 and 5). We reason that this increased uptake of ATIII correlates with thrombin production within the damaged subendothelium8 due to the expression of tissue factor25,26 at the site of injury and a liberal supply of plasma coagulation factors due to the absence of the endothelial barrier.

The rapid clearance of radiolabeled ATIII-β, compared with ATIII-α, from the plasma compartment27–29 was complemented by a rapid uptake of ATIII-β by the organ capillary systems studied (Table 1). Carlson et al.27 reported and we29 have confirmed that the fractional catabolic rate of rabbit ATIII-β in the vascular compartment and its fractional distribution in the extravascular compartment in the rabbit are substantially greater than corresponding values for ATIII-α. We infer from the comparative behavior of the isoforms in vivo and from the effects of thrombin in vitro (Figures 2 and 6) that the subendothelium-bound β isoform reacts more readily than α with thrombin on or within the vascular wall. Previously, we reported that aortabound unfractionated 125I-ATIII was displaced from the subendothelium by thrombin in vitro and that much of the displaced radioactivity consisted of high-molecular-weight components, presumably thrombin–ATIII complexes.6 With respect to Figure 6, the response of the individual subendothelium-bound isoforms to various concentrations of thrombin was compared. Clearly, the β isoform was displaced more rapidly than was α. We assume that the released radioactivity consisted of the respective thrombin–ATIII complexes although no attempt was made to identify these products.

Pretreatment of the de-endothelialized aorta (ballooned in vitro) with heparinase destroyed the subendothelial binding sites for the β isoform faster than those for α (Figure 5). From this result and from the behavior of ATIII-α and ATIII-β on heparin-Sepharose,30 we deduce that the isoforms bind to two different types of binding sites on heparan sulfate chains in the subendothelium, possibly a high-affinity site for the β isoform and a low-affinity site for α and β. Whether the two isoforms can compete for the low-affinity sites is not known.

From our studies of the rabbit aorta before and after a de-endothelializing injury, we propose the following model to explain the behavior of ATIII during hemostasis. When bound to extracelullar sites in the blood vessel subendothelium, ATIII is presumably located at a favorable site for inhibiting thrombin generated during injury.5,31 Thus, a low rate of thrombin production due to "wear-and-tear" injury may be contained by endogenous pericellular ATIII.
and other antithrombins (e.g., protease nexin). In contrast, a high rate of thrombin production resulting from a more severe injury may overcome the endogenous ATIII, and an appropriate measure of thrombus would result. Such a system requires that any thrombin–ATIII complex formed in the subendothelium be replaced with active ATIII at the heparan sulfate–binding sites to maintain the antithrombin potential of the extracellular space. For this reason, the rate of turnover of ATIII at the site of injury would be expected to increase. The ATIII–thrombin complexes either are endocytosed by vascular cells or pass into the blood stream or the lymph circulation. Whether the ATIII–α-thrombin and ATIII–β-thrombin complexes differ in their rates of clearance and catabolism is not known.

Finally, the pattern of behavior of the isoforms of ATIII parallel, to a large extent, those of another plasma glycoprotein, plasminogen (Plg). Plg also comprises two isoforms (designated 1 and 2), which contain dissimilar quantities of carbohydrate but which differ in their potential for activation to plasmin by various activators. Biosynthesis of each isoform is directed by a distinct mRNA. Both isoforms bind in a dose-dependent manner to U937 cells, but Plg 2, the less glycosylated isoform, binds with 10 times more affinity than does Plg 1. Human Plg 2 is cleared more rapidly from the circulation of the mouse than human Plg 1, and also rabbit Plg 2 is cleared more rapidly than Plg 1 from the rabbit circulation (E. Regoeczi and M.W.C. Hatton, unpublished results). Glycan heterogeneity is a common feature in glycoproteins, but diversity between the number of glycans contained by the isoforms of any glycoprotein is less well known. Tissue plasminogen activator and protein C are other examples of glycoproteins comprising two isoforms containing dissimilar numbers of glycans. Perhaps other plasma glycoproteins exist as two isoforms, of which one is relatively underglycosylated and possibly more active than the other isoform.

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Antithrombin III-beta associates more readily than antithrombin III-alpha with uninjured and de-endothelialized aortic wall in vitro and in vivo.

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