Inhibitory Effects of Antithrombin III against Leukocyte Rolling and Infiltration during Endotoxin-Induced Uveitis in Rats

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Purpose. This study was designed to investigate the suppressive effects of antithrombin (AT)III on inflammatory reactions during endotoxin-induced uveitis (EIU) in rats by studying leukocyte–endothelium interactions.

Methods. EIU was induced in Lewis rats by footpad injection of lipopolysaccharide (LPS). ATIII was administered immediately after or at 6 hours after LPS injection. Its suppressive effects on inflammatory leukocyte behavior were evaluated in vivo with acridine orange digital fluorography. Clinical signs of inflammation were also examined, and aqueous humor (AH) was collected to evaluate leukocyte infiltration and protein leakage. In a separate experiment, P-selectin mRNA expression was studied in the iris-ciliary body (ICB) and the retina.

Results. After treatment with ATIII, leukocyte rolling was substantially inhibited along the retinal veins, suppressing subsequent leukocyte infiltration into the vitreous cavity. Similarly, leukocyte infiltration and protein leakage into the AH were significantly reduced with ATIII treatment. In addition, delayed administration of ATIII after EIU induction significantly attenuated these inflammatory reactions. The levels of P-selectin mRNA expression in both ICB and retina, which were upregulated after LPS injection, were substantially lower in the ATIII-treated rats.

Conclusions. ATIII treatment significantly inhibited inflammatory reactions induced with LPS. Its suppressive effects on P-selectin expression could contribute to the attenuation of leukocyte infiltration, possibly by inhibiting leukocyte rolling. The current findings suggest that ATIII may have a role in the management of patients with uveitis. (Invest Ophthalmol Vis Sci. 2001;42:1553–1560)

Antithrombin (AT)III is an important endogenous inhibitor of serine proteases that are generated within the coagulation cascade. Clinically, it has been widely used as treatment for ATIII deficiency or disseminated intravascular coagulation. Recently, it has been demonstrated that ATIII may have other functions in addition to its role in inhibiting clotting. O’Reilly et al.1 have shown its remarkable antiangiogenic and antitumor activities. In addition, some investigators have reported that ATIII suppresses leukocyte infiltration and subsequent tissue damage in endotoxin-induced vascular injury,2 or ischemia–reperfusion (I/R) injury.3,4 In the field of septic shock, the suppressive effects of ATIII on inflammation have been highlighted.5,6 In patients with severe sepsis, treatment with ATIII improved lung function and prevented septic liver and kidney failure.7 Now, ATIII is attracting a great deal of attention as a possible treatment for these various kinds of conditions, because no adverse side effects have been reported in human trials.8 However, little information is available about the effects of ATIII on ocular inflammation and the mechanisms of its anti-inflammatory action.

When generated endogenously or administered intravenously, ATIII is tethered to the wall of the microvasculature with the help of heparin and is then bound to the active site on thrombin to suppress its functions. In addition to its hemostatic role, thrombin has the ability to induce interactions between endothelial cells and blood-flowing cells, such as leukocytes9–11 or tumor cells.12,13 Because these interactions are thought to be the initial step in leukocyte infiltration, tumor invasion, or metastasis,14,15 the anti-inflammatory and antiangiogenic properties of ATIII may be based partially on its suppressive effects on these interactions. In this study, we investigated the anti-inflammatory effects of ATIII during endotoxin-induced uveitis (EIU) in rats by studying leukocyte-endothelium interactions in vivo.

EIU is an animal model of ocular inflammation induced by subcutaneous injection of lipopolysaccharide (LPS).16,17 It is characterized by blood–ocular barrier disruption and leukocyte infiltration. At sites of inflammation, the recruitment of circulating leukocytes out of the vessels is thought to occur through a multistep cascade of leukocyte–endothelium interactions involving sequential rolling, firm adhesion, and transmigration into inflamed tissues.14,15,16–20 To investigate these interactions in vivo, intravital microscopy is a popular technique. In this technique, acridine orange (AO) is widely used to label leukocytes.21–20 By investigating the AO-stained leukocytes in eyes, we have clearly visualized and quantitatively evaluated each step of leukocyte recruitment in the retina and the vitreous during EIU.21–32 Although EIU in rats has been thought to be predominantly an anterior uveitis model, in vivo evaluation of the sequential leukocyte behavior in the posterior segment of eyes would be helpful to investigate the mechanism by which ATIII blocks the development of EIU.

During this multistep cascade of leukocyte infiltration, each process is mediated by distinct adhesion molecules and is regulated elaborately.14,15,18–20 Previous studies have demonstrated that monoclonal antibodies against these adhesion molecules can suppress leukocyte infiltration during EIU.33–38 We have shown that, among these adhesion molecules, P-selectin contributes to leukocyte rolling during EIU.39 Before mediating leukocyte rolling, P-selectin should be expressed on endothelial cells in response to a stimulator such as thrombin. There-
fore, we hypothesized that ATIII might downregulate P-selectin expression and thereby suppress leukocyte–endothelium interactions to block the development of EIU in rats. To verify our hypothesis, we evaluated P-selectin gene expression in the iris-ciliary body (ICB) and the retina.

METHODS

Animal Model

Animals were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. EIU was induced in female Lewis rats, each weighing approximately 200 g, by injecting 100 μg of LPS (Salmonella typhimurium; Difco Laboratories, Detroit, MI) diluted in 0.1 ml sterile saline into one hind footpad. Control animals received a footpad injection of saline alone.

Treatment

ATIII, obtained from Aventis Pharma (Frankfurt, Germany), was purified from heat-treated, pooled human plasma by absorption on fixed ATIII, obtained from Aventis Pharma (Frankfurt, Germany), was purified from heat-treated, pooled human plasma by absorption on fixed

ATIII concentrate used in this experiment.

To test the specificity of ATIII activity, inactivated ATIII, which was boiled at 100°C for 10 minutes, was administered immediately after LPS injection (n = 6).

AO Digital Fluorography

AO digital fluorography has been described elsewhere. In this technique, a scanning laser ophthalmoscope (SLO; Rodenstock Instruments, Munich, Germany) coupled with a computer-assisted image analysis system makes continuous high-resolution images of the fundus of animals injected with the metachromatic fluorescein AO (Wako Pure Chemical, Osaka, Japan). The obtained images are recorded on S-VHS videotape at the rate of 30 frames/sec for further analysis.

Immediately before AO digital fluorography was performed, rats were anesthetized with a 1:1 mixture of xylazine hydrochloride (4 mg/kg) and ketamine hydrochloride (10 mg/kg) and the pupils dilated with 0.5% tropicamide and 2.5% phenylephrine hydrochloride. In each rat, a catheter was inserted into the tail vein. Arterial blood pressure was monitored with a blood pressure analyzer (ITC, Woodland Hills, CA). The rat was then placed on a movable platform, and AO (0.1% solution in saline) was injected through the tail vein catheter. The fundus was observed with the SLO in the 40° field. The behavior of leukocytes can be observed within several seconds after AO infusion, because the dye circulation time is less than 10 seconds, and AO is so membrane-permeable that leukocytes can be stained shortly after its infusion. Once infused, AO permeates the tissues gradually. Therefore, AO was injected for 1 minute at a rate of 1 ml/min to investigate the behavior of leukocytes for a few minutes. At 20 minutes after the injection of AO, the fundus was observed again to evaluate leukocyte infiltration into the vitreous cavity.

AO is thought to be a mutagenic and carcinogenic agent, and phototoxicity also has been noted after exposure of AO to light. However, AO has been used to study leukocyte behavior in various organs, and no undesirable effect on microcirculation of the dye has been reported.

Image Analysis

The video recordings were analyzed with an image analysis system consisting of a computer equipped with a video digitizer (Radius, San Jose, CA). The video image was digitized in real time (30 frames/sec) to 640 horizontal and 480 vertical pixels with an intensity resolution of 256 steps.

Rolling leukocytes were defined as leukocytes that moved more slowly than free-flowing leukocytes. To evaluate the number of rolling leukocytes, a locus 1 disc diameter away from the center of the optic disc was picked up in each vessel. The number of rolling leukocytes passing through this locus for 1 minute was counted. This was defined as the number of rolling leukocytes in a vessel. We then calculated the average number of rolling leukocytes in all major veins. This was used as the number of rolling leukocytes in a given rat. The number of leukocytes infiltrating into the vitreous was determined by counting the number of fluorescent dots in the vitreous within a circle with a radius of 1 disc diameter from the center of the optic disc.

Vessel diameters were measured at 1 disc diameter from the center of the optic disc in monochromatic images recorded before AO injection. Each vessel diameter was calculated in pixels as the distance between the half-height points determined separately on each side of the density profile of the vessel image. Averages of the arterial and venous diameters were used as the arterial and venous diameters for each rat. After the experiment, each rat was killed with an overdose of anesthesia, and the study eye was enucleated to determine a calibration factor to convert values measured on a computer monitor (in pixels) into real values (in micrometers).

Clinical Scoring

Clinical scoring of EIU was performed using the classification of Hoekzema et al. The eyes were examined clinically with a slit lamp, and the uveitis was graded using the following scoring system: iris.
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RESULTS

Leukocyte Rolling

No rolling leukocytes were identified in control rats, but leukocyte rolling was observed in rats with EIU, but only in major retinal veins. The flux of rolling leukocytes in EIU rats increased gradually after LPS injection and reached its peak at 12 hours. The correlation between venous diameter and the number of rolling leukocytes in EIU at 12 hours after LPS injection is shown in Figure 2. The number of rolling leukocytes had no significant correlation with the diameter of the vein. Figure 3 shows the effects of ATIII on the flux of rolling leukocytes along the major retinal veins. ATIII administered immediately after LPS injection significantly inhibited leukocyte rolling, compared with untreated rats \( (P < 0.0001) \). The flux of rolling leukocytes in ATIII-treated rats was reduced by 89.6\% \( (P = 0.0062) \), 83.5\% \( (P < 0.0001) \), 72.7\% \( (P = 0.0090) \), and 53.1\% \( (P = 0.020) \) at 6, 12, 24, and 48 hours after LPS injection, respectively.

Leukocyte Infiltration into Vitreous Cavity

In the control rats, no leukocytes were observed in the vitreous. With the induction of EIU, the number of leukocytes that infiltrated the vitreous cavity was increased from 24 hours after EIU induction, and peaked at 48 hours (Fig. 4). However, leukocyte infiltration into the vitreous cavity was significantly suppressed with ATIII treatment \( (P = 0.0004) \). At 48 hours after LPS injection, the maximum number of leukocytes in the vitreous cavity was reduced by 56.3\% in ATIII-treated rats compared with untreated rats \( (P = 0.0086) \), Fig. 5).

Analysis of Leukocytes and Protein in Aqueous Humor

Aqueous humor (AH) was collected by anterior chamber puncture using a 27-gauge needle. For the leukocyte count, the sample was suspended with an equal volume of 0.4\% trypan blue stain solution, and the leukocytes were counted under a light microscope. The concentration of protein in AH was determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA) and dilution of bovine serum albumin (Sigma Chemical, St. Louis, MO) as a standard.

P-Selectin Gene Expression

To evaluate the effect of ATIII on P-selectin mRNA expression, the ICB and the retina were collected from the eyes of the following three groups: control rats, untreated rats receiving LPS, and rats treated with ATIII (250 U/kg) at the time of LPS injection. Total RNA was isolated from the pooled ICB or retina of both eyes of each rat, according to the acid guanidinium thiocyanate-phenol-chloroform extraction method. The extracted RNA was quantified, and 4 \( \mu \)g was used to make cDNA with a cDNA synthesis kit (First Strand; Pharmacia Biotech, Uppsala, Sweden). Polymerase chain reaction (PCR) was performed using the method of Saiki et al. with slight modification. The following conditions were used: denaturation, 95\°C for 30 seconds; annealing, 55\°C for 30 seconds; extension, 72\°C for 60 seconds. The reaction was initiated by adding two units of polymerase (Taq NA; Perkin Elmer-Cetus, Norwalk, CT), after which 30 cycles for P-selectin and 25 cycles for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were performed. The primers used in this experiment were GCAGACAACGAGCCCAAATAACA (sense) and AGTGCAGTGGAGTATGTCAGAG (antisense) for P-selectin and TGGCACAGTCAAGGCTGAGA (sense) and AGTGCAGTGGAGTATGTCAGAG (antisense) for GAPDH.54,55 Nucleotide sequencing and restriction pattern analysis confirmed that PCR products were derived from the target cDNA sequences. To quantify the P-selectin gene expression, PCR was performed in a semiquantitative manner. In this procedure, 2 \( \mu Ci \) of radiolabeled dCTP was added to the PCR reaction mixture. The PCR products were then electrophoresed, the bands excised, and the radioactivity incorporated in the DNA measured by Cerenkov scintillation counting.

Statistical Analysis

All values are expressed as mean \( \pm \) SEM. Student’s \( t \)-test with Welch’s correction was used to compare two groups. To compare three or more conditions, statistical analysis was performed by analysis of variance, with post hoc comparisons tested by Scheffe’s procedure. Spearman’s rank correlation was used to test correlation. Differences were considered statistically significant at \( P < 0.05 \).

Figure 2. Relationship between vessel diameter and number of rolling leukocytes along the major retinal veins in EIU at 12 hours after LPS injection. No significant correlation was detected.

Figure 3. Effect of ATIII on the flux of rolling leukocytes after EIU induction. ATIII was administered immediately after LPS injection. Values are mean \( \pm \) SEM (\( n = 6 \) at each time point in both groups). \(* P < 0.01 \), \( \dagger P < 0.05 \), compared with untreated rats.
Major Retinal Vessel Diameters

Figure 6 shows the changes in major retinal vessel diameters in untreated rats and ATIII-treated rats. Both arteries and veins showed substantial vasodilation after EIU induction. In ATIII-treated rats, however, vasodilation in arteries and veins was significantly suppressed, compared with that in untreated rats ($P < 0.0001$ for both arteries and veins). The diameters of major retinal arteries and veins, in ATIII-treated rats were reduced by 16.0% ($P = 0.0026$) and 29.2% ($P = 0.0015$), respectively, at 24 hours after LPS injection (Fig. 7).

Clinical Score

Clinical scores in untreated and ATIII-treated rats were studied at 24 hours after EIU induction. Rats treated with ATIII immediately after EIU induction showed significantly reduced signs of inflammation compared with untreated rats ($P < 0.0005$). Untreated eyes had a mean clinical uveitis score of $5.5 \pm 0.3$, whereas ATIII-treated eyes had a mean clinical score of $3.0 \pm 0.4$.

Leukocyte Infiltration and Protein Leakage into AH

Figure 8 shows the effects of ATIII administered immediately after LPS injection on leukocyte count and protein concentration in AH. In untreated rats, both leukocyte count and protein concentration in AH gradually increased after EIU induction and reached a peak level at 24 hours. In ATIII-treated rats, however, leukocyte count and protein concentration in AH were significantly lower than in untreated rats ($P < 0.0001$ for both leukocyte count and protein concentration). At 24 hours after LPS injection, ATIII treatment suppressed leukocyte infiltration in AH by 90.8% ($P = 0.00068$) and protein leakage by 77.4% ($P = 0.00033$).

Blood Pressure and Leukocyte Count in Peripheral Blood

We measured the mean arterial blood pressure and peripheral leukocyte count in both ATIII-treated and untreated groups. There were no significant differences between groups.

Effects of Inactivated ATIII

As shown in Table 1, inactivated ATIII, which was administered immediately after LPS injection, did not suppress leukocyte rolling in retinal veins, leukocyte infiltration into vitreous cavity.
cavity, vasodilation of retinal vessels, clinical score, leukocyte infiltration into AH, or protein leakage into AH.

**Effects of ATIII Administered at 6 Hours after LPS Injection**

Figure 9 shows the effects of ATIII administered 6 hours after LPS injection. Similar to the concomitant treatment with ATIII, delayed administration of ATIII significantly suppressed leukocyte rolling in retinal veins at 12 hours (P < 0.0001) and subsequent leukocyte infiltration into the vitreous cavity at 48 hours (P = 0.0006). Vasodilation in arteries and veins at 24 hours was also significantly suppressed in ATIII-treated rats (P = 0.0004 and P = 0.0006, respectively). As for the anterior uveitis, ATIII-treated rats showed significantly reduced signs of inflammation at 24 hours (P = 0.018). Moreover, leukocyte count and protein concentration in AH were significantly lower in ATIII-treated rats at the peak of the anterior uveitis (P = 0.0006 and P = 0.023).

**Gene Expression of P-Selectin**

The levels of gene expression were shown as a ratio to the average values of control rats (Fig. 10). At 6 hours after LPS injection, P-selectin mRNA expression was upregulated in both ICB and retina. ATIII treatment immediately after LPS injection significantly suppressed P-selectin mRNA expression in the ICB (P = 0.014) and the retina (P = 0.047).

**Discussion**

In the present study, we demonstrated that ATIII blocked leukocyte rolling along the major retinal veins and subsequent leukocyte infiltration into the vitreous cavity during EIU in rats. Although EIU is thought to be predominantly an anterior uveitis model, increasing numbers of studies have demonstrated leukocyte infiltration in the posterior segment of the eye.58,57–63 Moreover, direct observation of the fundus with SLO demonstrated remarkable vasodilation of retinal vessels in EIU,51,59 which had been reported to be very mild in a study using histologic examination.57 Although leukocyte behavior in the posterior segment might be somewhat different from that in anterior uveitis, it would be helpful to evaluate the sequential steps of leukocyte infiltration to understand the mechanism of anti-inflammatory drugs.

Accumulating evidence indicates that the recruitment of circulating leukocytes into inflamed tissue is mediated through a multistep cascade of events involving sequential rolling, firm adhesion, and transmigration.19 Among these processes of leukocyte infiltration, the present study showed that ATIII suppressed leukocyte rolling in retinal veins. Leukocyte rolling is the initial step and prerequisite for the subsequent steps resulting in leukocyte infiltration.19 In this study, the number of leukocytes infiltrating the vitreous cavity was also successfully reduced with ATIII treatment. Moreover, ATIII suppressed vasodilation in the retina. Its inhibitory effects on leukocyte infiltration would contribute to its suppressive effects on retinal vasodilation, because accumulated leukocytes can produce a large amount of nitric oxide. We have demonstrated that anti-P-selectin monoclonal antibody can suppress vasodilation in addition to its effect of suppressing leukocyte rolling and subsequent infiltration in EIU.

We also demonstrated that ATIII significantly inhibited P-selectin mRNA expression in the retina and ICB at 6 hours after EIU induction. P-selectin is a key mediator of leukocyte rolling along inflamed endothelial cells,64–66 and our previous study showed that P-selectin mediates leukocyte rolling in EIU as

**Table 1. Effects of Inactivated ATIII on Endotoxin-Induced Uveitis**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Time (h)</th>
<th>Untreated</th>
<th>Inactivated ATIII-Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flux of rolling leukocytes (min)</td>
<td>12</td>
<td>32.2 ± 3.2</td>
<td>34.6 ± 3.8</td>
</tr>
<tr>
<td>LEAKING LEUKOCYTES IN VITREOUS (\text{count/mm})</td>
<td>48</td>
<td>30.5 ± 4.3</td>
<td>29.2 ± 4.0</td>
</tr>
<tr>
<td>Venous diameter (\mu m)</td>
<td>24</td>
<td>29.3 ± 0.8</td>
<td>26.8 ± 1.0</td>
</tr>
<tr>
<td>Uveitis score</td>
<td>24</td>
<td>56.8 ± 1.8</td>
<td>58.8 ± 2.2</td>
</tr>
<tr>
<td>Leukocyte counts in AH (\mu m)</td>
<td>24</td>
<td>1718 ± 230</td>
<td>1791 ± 382</td>
</tr>
<tr>
<td>Protein concentration in AH (mg/mL)</td>
<td>24</td>
<td>17.2 ± 1.8</td>
<td>18.4 ± 1.0</td>
</tr>
</tbody>
</table>

No significant differences were observed between groups in any parameters measured.
well. The peak time point of P-selectin gene expression in the retina of EIU rat is reportedly at 6 and 24 hours after LPS injection, whereas leukocyte rolling in the veins reached a peak at 12 hours. Therefore, our finding of suppressed P-selectin mRNA expression could account for the reduced leukocyte rolling at 12 hours. ATIII would suppress leukocyte rolling by inhibiting the expression of P-selectin on the endothelial cells of retinal veins.

Furthermore, ATIII treatment substantially reduced leukocyte infiltration in the AH. Our experiment using PCR demonstrated that ATIII suppressed P-selectin mRNA expression in the ICB at 6 hours after EIU induction. The P-selectin expression in the iris vessels is reportedly prominent at from 5 to 7 hours after LPS injection, and leukocyte rolling in iris vessels has been shown to increase during EIU in rats. Therefore, the suppression of P-selectin mRNA expression could account for the anti-inflammatory effects of ATIII on anterior uveitis, partly through inhibition of leukocyte rolling in the anterior uveal vessels. Moreover, ATIII successfully suppressed protein leakage into AH. The attenuation of the process of leukocyte infiltration may contribute to the protective effects on blood-ocular barrier disruption during EIU as well as its suppressive effects on vasodilation in the retina.

Experimental autoimmune uveitis (EAU) is a model of chronic uveitis. We have demonstrated that rolling leukocytes can be observed in the major retinal veins during EAU. Also in EAU, adhesion molecules such as L-selectin, intercellular adhesion molecule-1, and lymphocyte function associated antigen-1 have been shown to play important roles during leukocyte infiltration, and therefore ATIII may have a role in the management of the more chronic type of inflammation by downregulating these adhesion molecules.

Among various studies on the suppressive effects of ATIII on LPS-induced inflammation both in vitro and in vivo, there is no report that ATIII binds LPS. The possibility that ATIII binds LPS cannot be denied, but ATIII would then exert its anti-inflammatory effects by inhibiting later conditions induced by LPS. We demonstrated that ATIII administered at 6 hours after LPS injection still had anti-inflammatory effects on EIU, and thus it may be that ATIII suppresses leukocyte infiltration during EIU by downregulation of P-selectin expression, rather than by binding LPS.

The method for ATIII purification has been established, and various effects of ATIII have been investigated using this method. The purity of the ATIII concentration used in this study was shown, and other antiproteases were below detectable levels. The anti-inflammatory effects observed in the present study could thus be said to originate from ATIII itself.

In contrast to the anti-inflammatory effects of ATIII at 12 and 24 hours after LPS injection, its effects were somewhat weakened at 48 hours. This could be explained by the half-life of ATIII, which in rats is reportedly 12 hours. When administered immediately after LPS injection, sufficient concentrations of ATIII could not be maintained for 48 hours. In support of this view, ATIII administered at 6 hours after LPS injection suppressed leukocyte infiltration into the vitreous cavity at 48 hours, more effectively than when administered immediately after LPS injection.

Clinically, it is valuable to know that ATIII has a significant inhibitory effect when administered after EIU has been established. ATIII has been reported to attenuate organ damage when administered after endotoxin challenge in animal models of endotoxemia. Furthermore, patients with severe sepsis who were treated with ATIII showed improvement of lung
function and did not experience septic liver and kidney failure. Accordingly, ATIII may be useful not only preventatively, but may also work therapeutically for ocular inflammations.

Today, steroids are widely used in the treatment of uveitis. Similar to ATIII, steroids are endogenous agents that have various effects. However, their adverse side effects can cause serious problems. In contrast, ATIII has been used widely and safely in humans and may thus be useful when used in conjunction with other treatments for uveitis.

References

42. Emerson T Jr, Fournel MA, Redens TB, Taylor F Jr. Efficacy of antithrombin III supplementation in animal models of fulminating