Inhibitory Effects of Antithrombin III on Interactions between Blood Cells and Endothelial Cells during Retinal Ischemia–Reperfusion Injury

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Purpose. Infiltrating leukocytes have long been widely thought to be key mediators of ischemia–reperfusion injury. Recently, however, evidence suggests that platelets accumulating in postischemic tissues also contribute to ischemia–reperfusion injury because of their inflammatory properties and promotion of formation of thrombi. This study was designed to evaluate quantitatively the inhibitory effects of antithrombin (AT)-III on the interactions between blood cells and retinal endothelial cells in vivo after transient retinal ischemia.

Methods. Transient retinal ischemia was induced for 60 minutes in male Long-Evans rats by ligation of the optic nerve. AT III (250 U/kg) was administered intravenously just after induction of ischemia. Leukocyte and platelet behavior in the retina was evaluated in vivo with a scanning laser ophthalmoscope. Expression of P-selectin and intracellular adhesion molecule (ICAM)-1 in the postischemic retina was investigated by reverse transcription–polymerase chain reaction and immunohistochemistry. After 14 days of reperfusion, ischemia-induced retinal damage was evaluated histologically.

Results. Administration of AT III significantly inhibited leukocyte rolling along the major retinal veins and subsequent accumulation of leukocytes in the postischemic retina. Furthermore, the maximum number of rolling and adherent platelets was reduced by 76% ($P < 0.01$) and 48% ($P < 0.01$), respectively, at 12 hours after reperfusion. Immunohistochemical studies also revealed the suppressive effect of AT III on expression of P-selectin and ICAM-1. Finally, histologic examination demonstrated the protective effects of AT III against retinal damage after transient retinal ischemia.

Conclusions. This study demonstrates the inhibitory effects of AT III on leukocyte and platelet recruitment to the postischemic retina, which may account for the neuroprotective properties of this α-2 globulin against retinal ischemia–reperfusion injury. (*Invest Ophthalmol Vis Sci.* 2003;44:332–341) DOI: 10.1167/iovs.02-0493

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Infiltrating leukocytes have long been acknowledged to be a feature of ischemia–reperfusion injury.[1–4] Recently, however, evidence suggests that platelets also play an important role in the pathogenesis of ischemia–reperfusion injury.[5,6] The importance of platelets is supported by many studies that have demonstrated the beneficial effects of platelet depletion against ischemia–reperfusion injury.[7–9] Platelets are known to participate in postischemic tissue damage not only through formation of thrombus, but also through the release of free radicals and cytokines.[10–14] In addition, platelets have been reported to recruit leukocytes to ischemic sites through the expression of adhesion molecules on their surface or by the release of cytokines.[15–19] Moreover, platelets can modulate leukocyte functional responses.[20,21]

Thrombin, which is the terminal serine protease of the coagulation cascade, has the ability to activate platelets and fibrinogen. Recently, many investigators have focused on the role of thrombin in various pathologic conditions. It has been demonstrated that an increase in thrombin in postischemic tissues activates vascular endothelial cells. Such activated vascular endothelial cells express adhesion molecules, which contribute to the recruitment of leukocytes and platelets.[22–24] Antithrombin (AT)-III is an α-2 globulin that inhibits thrombin activity. Recently, Ostrovsky et al.[25] reported that AT-III protects against ischemia–reperfusion injury by suppressing the leukocyte–endothelium interaction in postischemic tissues. To our knowledge, however, little information is available about the effect of AT-III on platelet behavior in ischemia–reperfusion injury.

We recently developed an in vivo method to quantitatively evaluate platelet-endothelium interactions in rat retina.[26] Using this method, we have found that platelets roll along and adhere to retinal venous endothelium during ischemia–reperfusion and that these interactions are mediated by endothelial P-selectin, not by platelet P-selectin.[27] In the study described herein, we evaluated the effects of AT-III on leukocyte– and platelet–endothelium interactions and on the expression of adhesion molecules in postischemic rat retina.

Materials and Methods

Animal Model

Male pigmented Long-Evans rats (200–250 g) were used in this study. Transient retinal ischemia was induced for 60 minutes in the right eye of each rat.[28,29] Rats were anesthetized with a mixture (1:1) of xylazine hydrochloride (4 mg/kg) and ketamine hydrochloride (10 mg/kg). The pupils were dilated with 0.5% tropicamide and 2.5% phenylephrine hydrochloride. After lateral conjunctival peritomy and disinsertion of the lateral rectus muscle, the optic nerve of the right eye was exposed by blunt dissection. A 6-0 nylon suture was passed around the optic nerve and tightened until blood flow ceased in all the retinal vessels. Complete nonperfusion was confirmed by visualization through an operating microscope. After 60 minutes of ischemia, nonperfusion was

confirmed through an operating microscope, and the suture was removed, and reperfusion of the vessels was observed through the microscope.

AT-III was obtained from Aventis Pharma (Frankfurt, Germany). AT-III–treated rats were injected intravenously with 250 U/kg of AT-III just after induction of ischemia.[25,30] Vehicle-treated rats were given the same volume of saline. All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Acridine Orange Digital Fluorography

Acridine orange (AO) fluorography has been described in detail elsewhere.[31,32] 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 fundus stained by the metachromatic fluorochrome AO (Wako Pure Chemicals, Osaka, Japan). This dye emits a green fluorescence when it interacts with DNA. The spectral properties of AO-DNA complexes are similar to those of sodium fluorescein, with an excitation maximum at 502 nm and an emission maximum at 522 nm. The argon blue laser was used for the illumination source, with a regular emission filter normally used in fluorescein angiography. The obtained images were recorded for further analysis on an S-VHS videotape at the video rate of 30 frames/sec.

Evaluation of Leukocyte–Endothelial Cell Interaction

AO digital fluorography was performed at 6, 12, 24, and 48 hours after reperfusion in both the AT-III–treated and vehicle-treated groups. Nonischemic rats were evaluated as the control. Six different rats were used at each time point in each group.

Immediately before AO digital fluorography, each rat was anesthetized, and the pupil of the right eye was dilated as described for the induced ischemia phase. A contact lens was used to retain corneal clarity throughout the experiment. Each rat had a catheter inserted into the tail vein and was placed on a movable platform. Arterial blood pressure and heart rate were monitored with a blood pressure analyzer (IITC, Woodland Hills, CA). AO (0.1% solution in saline) was injected continuously through the catheter for 1 minute at a rate of 1 mL/min. The fundus was observed with the SLO in the 40° field for 5 minutes. At 30 minutes after injection of AO, the fundus was observed again, to evaluate leukocyte accumulation in the retinal microcirculation.

Blood Sampling and Platelet Preparation

Carboxyfluorescein diacetate succinimidyl ester (CFDASE; Molecular Probes, Eugene, OR) is a nonfluorescent precursor that diffuses into cells and forms a stable fluorochrome carboxyfluorescein succinimidyl ester (peak absorbance 492 nm, peak emission 518 nm) after being catalyzed by esterase. This enzymatic reaction occurs predominantly in leukocytes and platelets and partially in serum. Intracellular fluorophores react with lysine residues of protein and remain within the cell as long as the membrane is intact.[33]

CFDASE was dissolved in dimethyl sulfoxide (Wako Pure Chemicals) to a concentration of 15.6 mM, and small aliquots (150 μL) were stored at −70°C until use. Platelet samples were prepared in accordance with the method described previously.[26,27] In brief, blood samples from donor rats were harvested from the abdominal artery and collected in polypropylene tubes containing a 2-mL volume of acid-citrate-dextrose (38 mM citric acid, 75 mM trisodium citrate, and 100 mM dextrose).[34] The blood was centrifuged at 250*g* for 10 minutes. Platelet-rich plasma was then gently transferred to a fresh tube and centrifuged at 2000*g* for 10 minutes. The platelet pellet was resuspended in 10 mL of Hanks' balanced salt solution (HBSS; Gibco, Grand Island, NY) and incubated with 150 μL of CFDASE solution for 30 minutes at 37°C. After incubation, the platelet suspension was centrifuged again at 2000*g* for 10 minutes. The pellet was resuspended in 10 mL of HBSS and centrifuged again at 2000*g* for 10 minutes,[34] after which the platelet pellet was resuspended in HBSS at a concentration of 6×10^8 platelets/0.2 mL.

Evaluation of Platelet–Endothelial Cell Interactions

Platelet behavior in the retinal microcirculation was evaluated at 6, 12, 24, and 48 hours after reperfusion. Nonischemic rats were used as controls. Immediately before platelet administration, rats were anesthetized with the agents described earlier. Six rats were used at each time point. A total of 6×10^8 platelets were infused, to evaluate their interactions with the retinal endothelial cells. The fundus was observed by SLO with the argon blue laser and a regular emission filter typically used for fluorescein angiography. The obtained images were recorded on an S-VHS videotape for further analysis.

Image Analysis

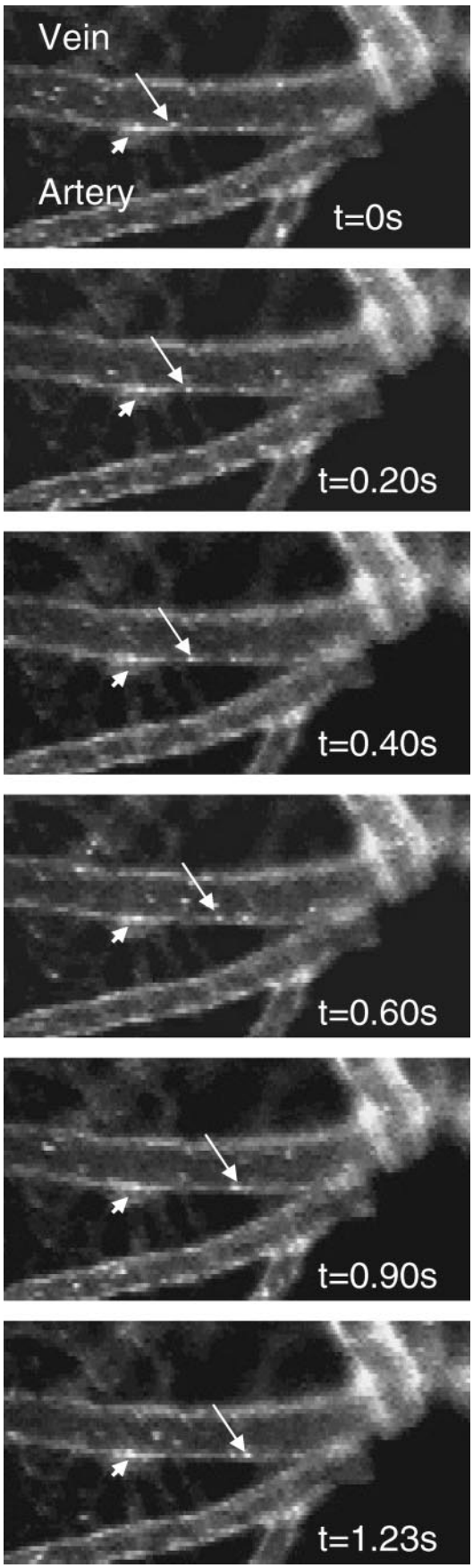
Rolling leukocytes were defined as leukocytes that moved at a velocity slower than that of free-flowing leukocytes. The number of rolling leukocytes was calculated as the total number crossing a fixed area of each of the veins at a distance 1 disc diameter from the center of the optic disc. For evaluating accumulated leukocytes, an observation area around the optic disc measuring five disc diameters in radius was outlined by drawing a polygon bordered by the adjacent major retinal vessels. The area was measured in pixels and the density of trapped leukocytes was calculated by dividing the number of static leukocytes, which were recognized as fluorescent dots, by the area of the observation region.

Rolling platelets were defined as platelets that moved at a slower velocity than free-flowing platelets in a given vessel and that made intermittent adhesive contact with vascular endothelial cells. The number of rolling platelets in each major retinal vein was calculated as the number of rolling platelets observed along each vein for 1 minute at a distance 1 of disc diameter from the optic disc center. Their numbers are given as platelets per venous diameter. The averages of the individual numbers were used as the number of rolling platelets for each rat. A platelet was defined as adherent to vascular endothelium if it remained stationary for longer than 10 seconds. Adherent platelets were calculated as the total number of adherent platelets along all major retinal veins observed for 1 minute within a circle with a radius of 500 μm from the center of the optic disc. The number was expressed as the number of adherent platelets per square millimeter of

Table 1. Mean Arterial Blood Pressure and Peripheral Leukocytes

	Control	6 h	12 h	24 h	48 h
Vehicle-Treated Retina					
MABP (mm Hg)	117.0 ± 4.2	115.4 ± 8.4	121.5 ± 9.4	111.8 ± 6.0	103.8 ± 9.2
WBC ($\times 10^3/\mu$L)	6.2 ± 0.4	10.8 ± 1.1	12.4 ± 1.4	9.2 ± 0.3	8.8 ± 0.6
AT-III–Treated Retina					
MABP (mm Hg)	117.0 ± 4.2	101.8 ± 4.0	120.0 ± 5.7	108.8 ± 7.9	108.0 ± 6.0
WBC ($\times 10^3\mu$L)	6.2 ± 0.4	10.3 ± 1.2	11.2 ± 0.7	9.9 ± 0.6	9.3 ± 1.0

No significant differences were found between vehicle-treated and AT-III–treated rats.



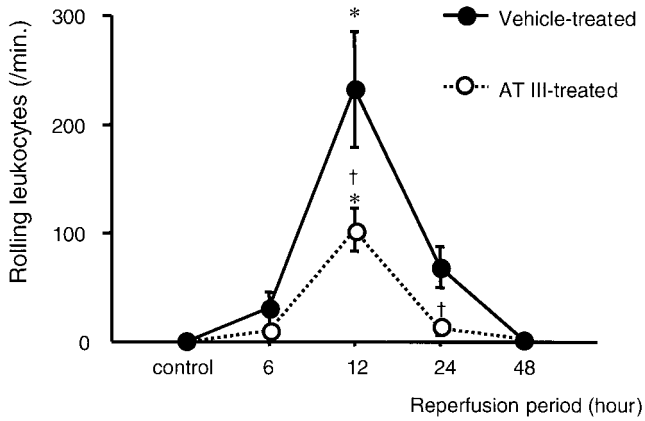


FIGURE 2. Inhibitory effect of AT-III on the flux of rolling leukocytes in postischemic retina. Data are expressed as the mean ± SEM ($n = 6$ at each time point in both groups). $^*P < 0.05$ compared with data in control rats in each strain. $\dagger P < 0.05$ compared with data in vehicle-treated rats.

the endothelial surface of the major retinal veins. All parameters were evaluated after a stabilization period of 5 minutes after the administration of platelets.

Leukocyte and Platelet Count in Peripheral Blood

Blood anticoagulated with EDTA was obtained from the abdominal artery of each rat after the experiment. The blood sample was analyzed by a hematology analyzer (ERMA, Tokyo, Japan).

Semiquantification of P-selectin and ICAM-1 Gene Expression by Reverse Transcription–Polymerase Chain Reaction Method

To evaluate the effect of AT-III on P-selectin and intercellular adhesion molecule (ICAM)-1 mRNA expression, eyes were enucleated at 12 and 24 hours after reperfusion. Six rats were used at each time point. Each enucleated eye was cut into two pieces along the limbus, and then the retina was collected from the posterior segment. Nonischemic eyes were used as the control. Total RNA was isolated from the retina according to the acid guanidinium thiocyanate-phenol-chloroform extraction method.[35] The extracted RNA was quantified, and then 5 μg of the RNA was used to make cDNA. cDNA was synthesized with a kit (First Strand cDNA Synthesis; Pharmacia Biotech, Uppsala, Sweden). RT-PCR was performed by using the method of Saiki et al.,[36] with slight modification. The following conditions were used: denaturation at 94°C for 30 seconds, annealing at 55°C for 1 minute, and polymerization at 72°C for 1 minute. The reaction was performed for 35 cycles for P-selectin and ICAM-1 and 25 cycles for β-actin. The primers were CAAGAGGAACAACCAGGACT (sense) and AATGGCTTCACAGGTTGGCA (antisense) for P-selectin, AGACACAAGCAAGAGAAGAA (sense) and GAGAAGCCCAAACCCGTATG (antisense) for ICAM-1, and AGCTGAGAGGGAAATCGTGC (sense) and ACCAGACAGCACTGTGTTGG (antisense) for β-actin. Expression of β-actin was used as the internal standard. Nucleotide sequencing and restriction pattern analysis confirmed that PCR products were derived from the target cDNA sequences. To quantify the P-selectin and ICAM-1 gene expression, PCR was performed in a semiquantitative manner.[37] In this procedure, 2 μCi of radiolabeled dCTP was added to the PCR reaction mixture. The PCR products were then electrophoresed, the bands excised, and the

FIGURE 1. Fundus images with AO digital fluorography in postischemic retina at 12 hours after reperfusion. Among free-flowing leukocytes, rolling leukocytes were observed along the major retinal veins (*arrows*). Some leukocytes adhered to the venous wall (*arrowheads*).

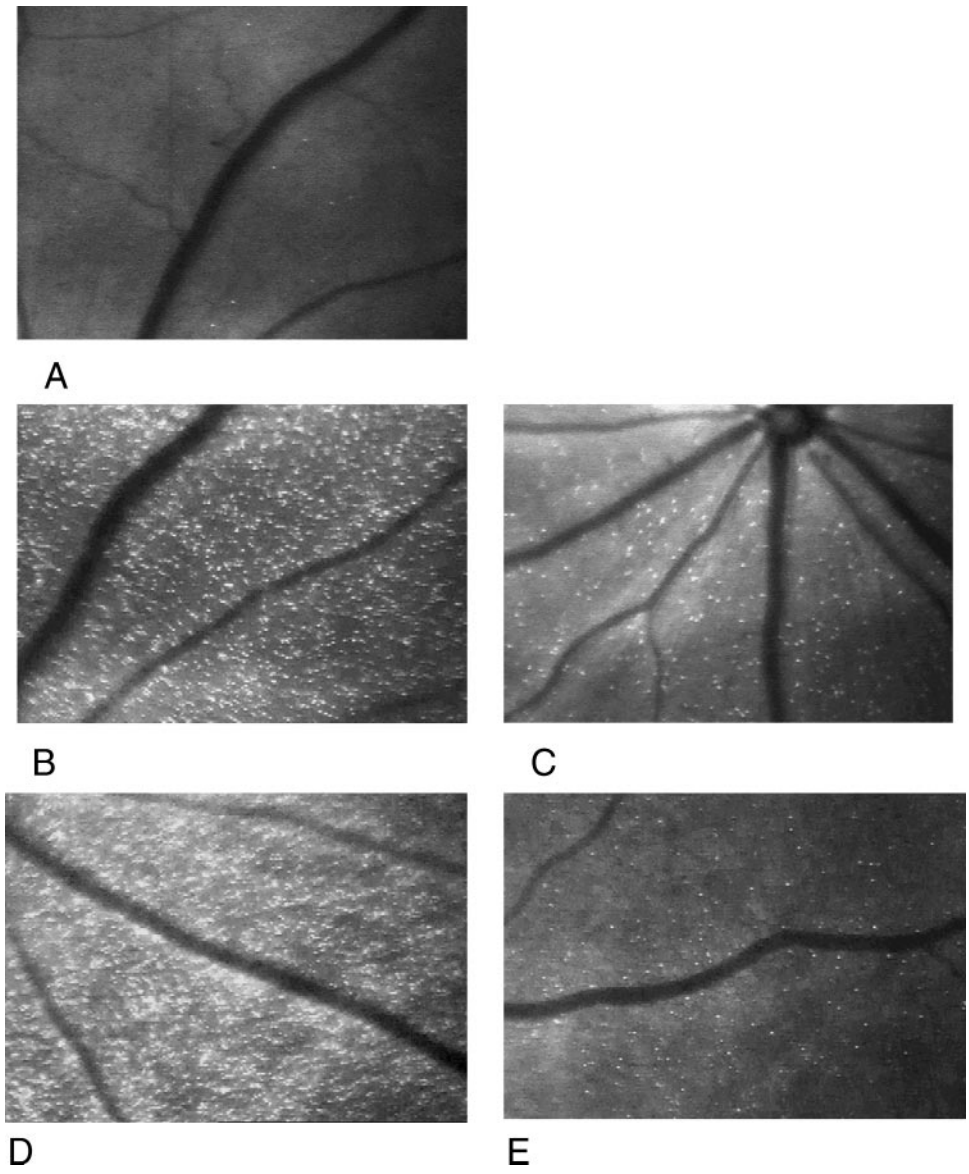


FIGURE 3. Fundus images with AO digital fluorography in postischemic and control retinas. Leukocytes accumulated in retinal microcirculation appeared as fluorescent dots at 30 minutes after AO injection. (**A**) Control retina, (**B**) vehicle-treated retina at 12 hours after reperfusion, (**C**) AT-III–treated retina at 12 hours after reperfusion, (**D**) vehicle-treated retina at 24 hours after reperfusion, (**E**) AT-III–treated retina at 24 hours after reperfusion.

radioactivity incorporated to the DNA measured by Cerenkov scintillation counting.

Immunohistochemical Procedures

To determine the expression of P-selectin and ICAM-1 in retinal veins, immunohistochemical study using specific antibodies was performed with rat retina. At 12 hours after reperfusion, rats were perfusion-fixed with 4% paraformaldehyde and 0.1 M PBS before enucleation. Subsequently, the enucleated eyes were further fixed for 2 hours at 4°C in 4% paraformaldehyde and 0.1 M PBS and washed for 5 minutes in PBS. Under a dissecting microscope, each enucleated eye was cut into two pieces along the limbus, and then incubated overnight at 4°C in 15% sucrose and 0.1 M PBS with gentle shaking. The eyecups were embedded in optimal cutting temperature compound (Tissue-Tek; Miles, Inc., Elkhart, IN) and frozen on powdered dry ice. Sections (10 μm) were cut on a cryostat and collected onto silanized slides (Dako Japan, Kyoto, Japan).

Retinal sections were washed twice for 3 minutes each in PBS and then incubated in 5% skim milk with 10% normal goat serum in PBS (blocking solution) for 30 minutes and a solution of antibodies to P-selectin (ARP2-4, 1:1000 dilution in blocking solution; Sumitomo Pharmaceuticals, Osaka, Japan) and ICAM-1 (1A29, 1:20 dilution in

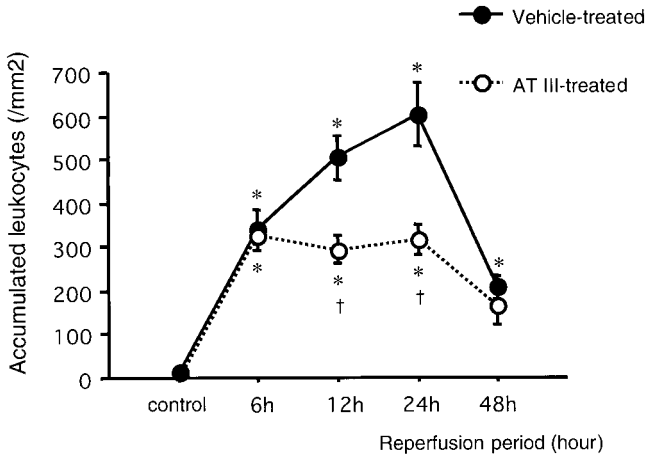


FIGURE 4. Inhibitory effect of AT-III on number of accumulated leukocytes in postischemic retina. Data are expressed as the mean ± SEM. *$P < 0.01$ compared with data in of control rats in each strain. †$P < 0.01$ compared with data in vehicle-treated rats.

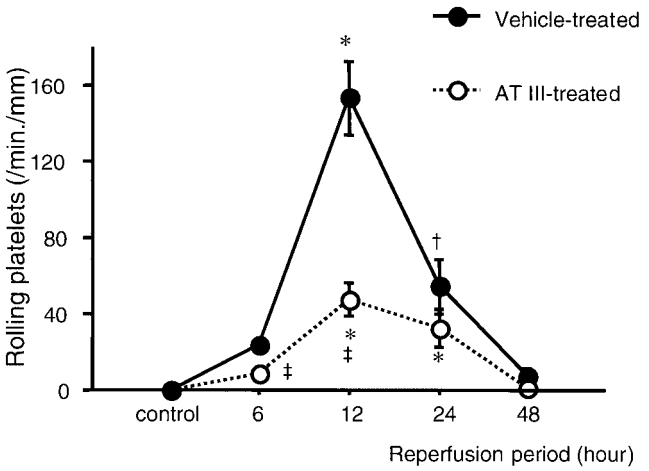


FIGURE 5. Inhibitory effect of AT-III on the flux of rolling platelets in postischemic retina. Data are expressed as the mean ± SEM. $^*P < 0.01$ and $\dagger P < 0.05$ compared with data in control rats in each strain. $\ddagger P < 0.01$ compared with data in vehicle-treated rats.

blocking solution; Serotec, Raleigh, NC) overnight at 4°C. After washing in PBS, they were treated for 30 minutes with Cy5-conjugated secondary antibodies (Chemicon, Temecula, CA) diluted 1:100. For double staining with von Willebrand factor (vWF) as a marker for vascular endothelium, the sections were incubated with a solution of antibody to vWF (1:400 dilution in blocking solution; Dako Japan) and Cy3-conjugated secondary antibody (Chemicon). Negative control sections without primary antibody were processed under the same conditions. Sections were mounted in 90% glycerol and 10% PBS and then observed under a confocal microscope (LSM410; Carl Zeiss, Oberkochen, Germany).

Histologic Procedures

Six eyes of six rats in the AT-III–treated, vehicle-treated, and sham-operation control groups were obtained to evaluate the severity of retinal damage. After 14 days of reperfusion, the rats were killed with an overdose of anesthesia. The surgically altered eyes were immediately enucleated and fixed in 1.48% formaldehyde and 1% glutaraldehyde in phosphate buffer and in 3.7% formaldehyde afterward. The eyes were then dehydrated, embedded in paraffin, sectioned with a microtome at 2-μm thickness, and stained with hematoxylin and eosin. Each section was cut along the horizontal meridian of the eye through the optic nerve head, perpendicular to the retinal surface. Retinal sections were examined with an optical microscope (×400) by using a masking procedure and then digitized by a charge-coupled device camera on a computer monitor.

To quantify the retinal damage induced by ischemia–reperfusion injury, we measured changes in thickness of the retina, using the method described by Hughs,[38] with slight modification.[39,40] The thicknesses of the inner plexiform layer (IPL), inner nuclear layer (INL), outer nuclear layer (ONL), and the overall retina from outer to inner limiting membrane (ILM-OLM) were measured. The thickness of the retinal layers in each section was measured at a distance of 1.5 mm from the center of the optic nerve head. The thickness recorded for each retinal layer was the average of 10 measurements of four sections from each eye.

Statistical Analysis

Data are reported as the mean ± SEM. The data were analyzed by one-way analysis of variance, using a post hoc test with Fisher's protected least-significance procedure. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Blood Pressure and Leukocyte Count in Peripheral Blood

Table 1 indicates the physiological variables for both AT-III– and vehicle-treated rats during the experiment. There were no significant differences between groups in mean arterial blood pressure (Table 1, MABP) throughout the course of ischemia–reperfusion injury. Leukocyte (Table 1, WBC) count in the peripheral blood substantially increased and reached a peak at 12 hours after reperfusion in both groups, but there was no significant difference in leukocyte count between groups.

Leukocyte–Endothelial Cell Interactions in Postischemic Retina

Immediately after the AO solution was infused intravenously, leukocytes were stained selectively among circulating blood cells. Nuclei of vascular endothelial cells were also stained. Among many free-flowing leukocytes labeled with AO, some were rolling slowly along the major retinal veins. In the major retinal arteries, no rolling leukocytes were observed throughout the experiments (Fig. 1). In vehicle-treated rats, the number of rolling leukocytes substantially increased after reperfusion and reached a peak at 12 hours, but the flux of rolling leukocytes decreased to almost basal levels at 48 hours after reperfusion. In AT-III–treated rats, leukocyte rolling was significantly inhibited (Fig. 2, $P = 0.0018$). The numbers of rolling leukocytes in AT-III–treated rats was significantly reduced by 56% ($P = 0.014$) and 83% ($P = 0.0079$) at 12 and 24 hours, respectively, after reperfusion compared with those in vehicle-treated rats. In the control rats, no leukocytes were observed rolling along the major retinal veins.

Figure 3 shows the changes in the numbers of accumulated leukocytes in the postischemic retinal microcirculation. In control rats, only a few leukocytes were recognized in the retinal microcirculation, whereas accumulated leukocytes began to increase in the ischemia groups at 6 hours after reperfusion and reached a peak at 24 hours. However, leukocyte accumulation in the postischemic retina was significantly inhibited with treatment by AT-III ($P = 0.0011$, Fig. 4). The number of accumulated leukocytes in postischemic retinas was reduced by 42% ($P = 0.0086$) and 47% ($P = 0.0017$) at 12 and 24 hours after reperfusion, respectively, compared with that in vehicle-treated rats.

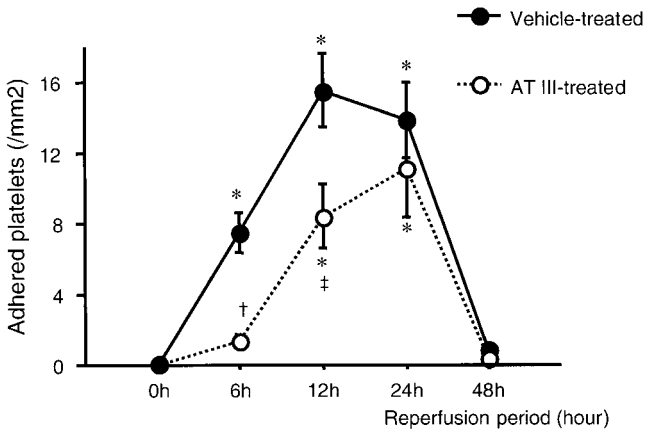


FIGURE 6. Inhibitory effect of AT-III on the number of adherent platelets in postischemic retina. Data are expressed as the mean ± SEM. $^*P < 0.01$ compared with data in control rats in each strain. $\dagger P < 0.01$ and $\ddagger P < 0.05$ compared with data in vehicle-treated rats.

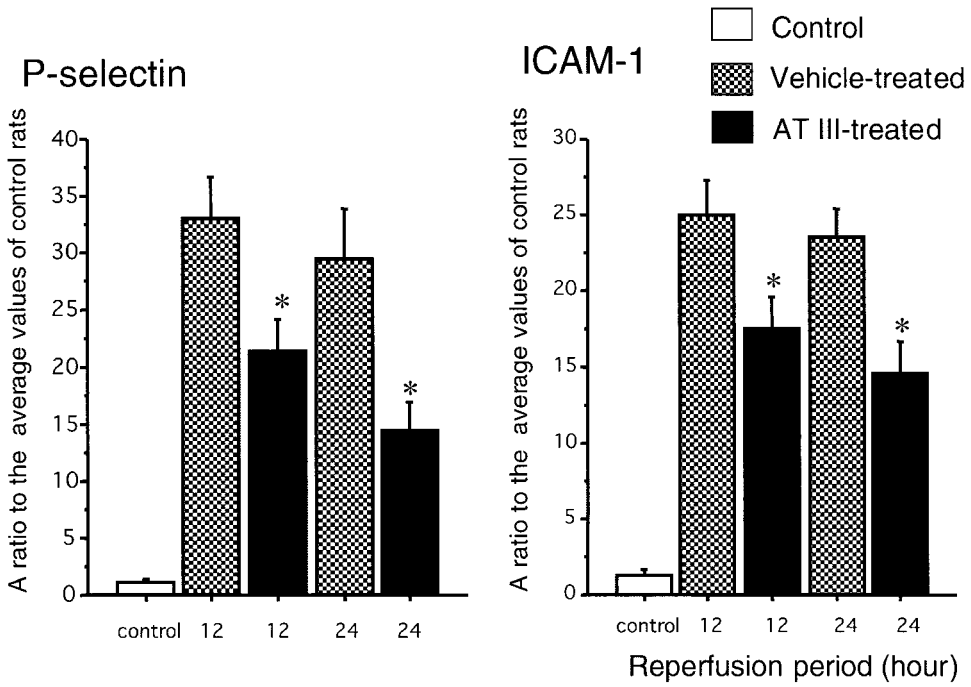


FIGURE 7. Inhibitory effect of AT-III on P-selectin (*left*) and ICAM-1 (*right*) gene expression in postischemic retina at 12 and 24 hours after reperfusion. Data are expressed as the mean ± SEM. $^*P < 0.05$ compared with data in vehicle-treated rats.

Platelet-Endothelial Cell Interactions in Postischemic Retina

Immediately after the labeled platelets were infused intravenously, they were visible as distinct fluorescent dots circulating in the retinal microcirculation. In the control rats, no platelets actively interacted with retinal endothelial cells. In the postischemic retina, however, some platelets were observed slowly rolling along major retinal veins among many free-flowing platelets. The number of rolling platelets along the major retinal veins increased substantially after reperfusion and reached a peak at 12 hours, then decreased at 48 hours almost to basal level. However, platelet rolling in postischemic retinas was suppressed significantly by treatment with AT-III ($P = 0.0001$, Fig. 5). At 12 hours after reperfusion in AT-III–treated rats, the number of rolling platelets was reduced by 69% ($P = 0.0008$), compared with that in vehicle-treated rats. Platelets showed minimal interaction with arterial endothelial cells in the postischemic retina throughout the experiment.

Most platelets rolling along the postischemic retinal veins were observed away from the optic disc or flowing downstream. Others were rolling at decreased velocity and were adhering to the vascular walls. The number of adherent platelets along major retinal veins increased substantially in the postischemic retinas and reached a peak at 12 hours after reperfusion, then decreased at 48 hours almost to basal level. Platelet adhesion in postischemic retinas was also significantly suppressed by treatment with AT-III ($P = 0.033$, Fig. 6). The AT-III treatment reduced the number of adherent platelets by 46% ($P = 0.025$) at 12 hours after reperfusion, compared with that in vehicle-treated rats.

Gene Expression of P-Selectin and ICAM-1 in the Retina

The levels of gene expression of P-selectin and of ICAM-1 in treated rats were shown as a ratio to the average values of control rats (Fig. 7). At 12 and 24 hours after reperfusion, P-selectin mRNA expression was upregulated substantially in postischemic retinas. AT-III treatment significantly suppressed P-selectin mRNA expression at both 12 (35%, $P = 0.024$) and at 24 hours (51%, $P = 0.012$) after reperfusion. ICAM-1 mRNA expression was also upregulated at 12 and 24 hours after reperfusion in postischemic retinas, but with treatment by AT-III, gene expression of ICAM-1 was reduced by 30% ($P = 0.036$) and 39% ($P = 0.010$), respectively, compared with vehicle-treated rats.

Immunostaining Studies

Immunostaining for P-selectin, ICAM-1, and vWF was performed in retinal specimens from sham-operation, vehicle-treated, and AT-III–treated rats. The immunostaining was absent on all sections incubated without the primary antibody. On sections incubated with vWF antibody, immunoreactivity was predominantly present in the venous endothelium of the retina in all rats. Intense P-selectin immunoreactivity was present in the venous endothelium from vehicle-treated rats, whereas faint immunoreactivity was seen in that from AT-III–treated rats (Fig. 8). We also observed that ICAM-1 was downregulated in the endothelium from AT-III–treated rats in comparison with that from vehicle-treated rats.

Histologic Study

Histologic examination showed destruction of the retinal structures in the sham-operation rats (Fig. 9). Thickness of the ILM-OLM in vehicle-treated rats was reduced to 59% that in control rats at 14 days after reperfusion. Figure 10 shows the thickness of each retinal layer at 14 days after reperfusion. The retinal structure of the postischemic retina was better preserved in AT-III–treated rats. With treatment by AT-III, retinal damage during retinal ischemia–reperfusion injury was reduced by 62% ($P = 0.0001$). The decrease in retinal thickness was more severe in the inner retina than in the outer retina. Thicknesses of the IPL and INL in vehicle-treated rats were reduced to 30% and 62%, respectively, of that in control rats at 14 days after reperfusion ($P = 0.0001$). With treatment by AT-III, however, retinal damage was reduced by 70% and 87% in the IPL and INL, respectively, compared with that in vehicle-treated rats ($P = 0.0001$).

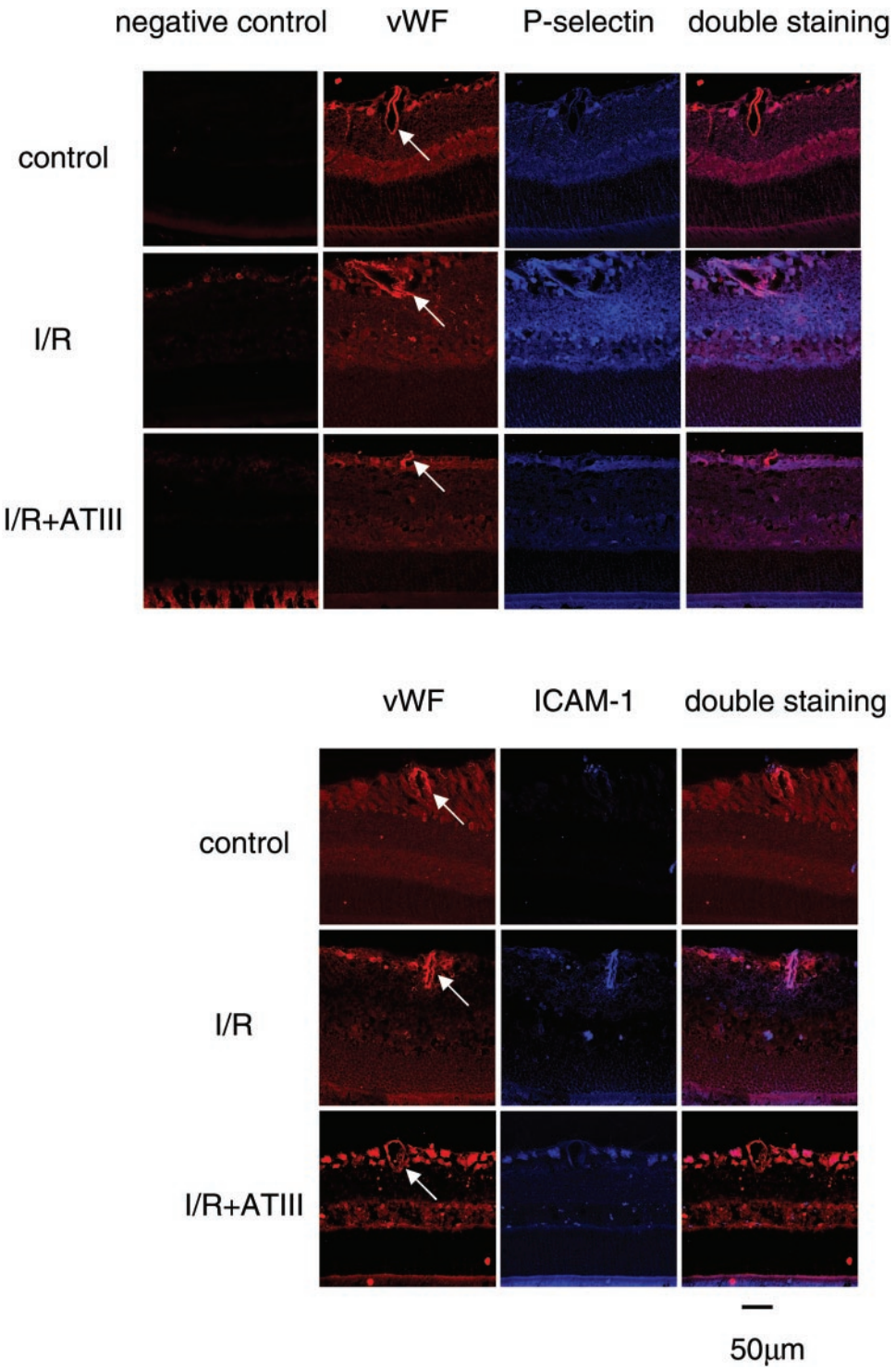


FIGURE 8. Immunostaining of frozen sections of retina for vWF, P-selectin, and ICAM-1. On sections without primary antibody, no immunoreactivity was seen in the venous endothelium. *Arrows*: venous staining. Compared with the vehicle-treated retina, P-selectin immunoreactivity was decreased in AT-III–treated retina. The suppression of ICAM-1 expression occurred in venous endothelium of AT-III–treated retina. Original magnification, ×400.

DISCUSSION

The interactions between blood cells and vascular endothelial cells are thought to be an initial step in the recruitment of leukocytes or platelets into the inflamed tissues, which leads to further tissue damage after transient retinal ischemia. In this study, we investigated the inhibitory effect of AT-III on these interactions during retinal ischemia–reperfusion injury. Administration of AT-III just after induction of ischemia significantly inhibited leukocyte rolling along postischemic retinal veins and subsequent accumulation of leukocytes in the retinal microcirculation. In addition, AT-III significantly suppressed platelet–endothelial cell interactions along the major retinal veins during reperfusion. The suppressed expression of P-selectin and ICAM-1 in AT-III–treated rats would account for the inhibitory effects of AT-III on the interactions between blood cells and vascular endothelial cells in postischemic retina. In the present study, we also demonstrated that AT-III substantially reduced retinal damage during retinal ischemia–reperfusion injury.

We have demonstrated that the number of accumulated leukocytes in the retina increases significantly after transient

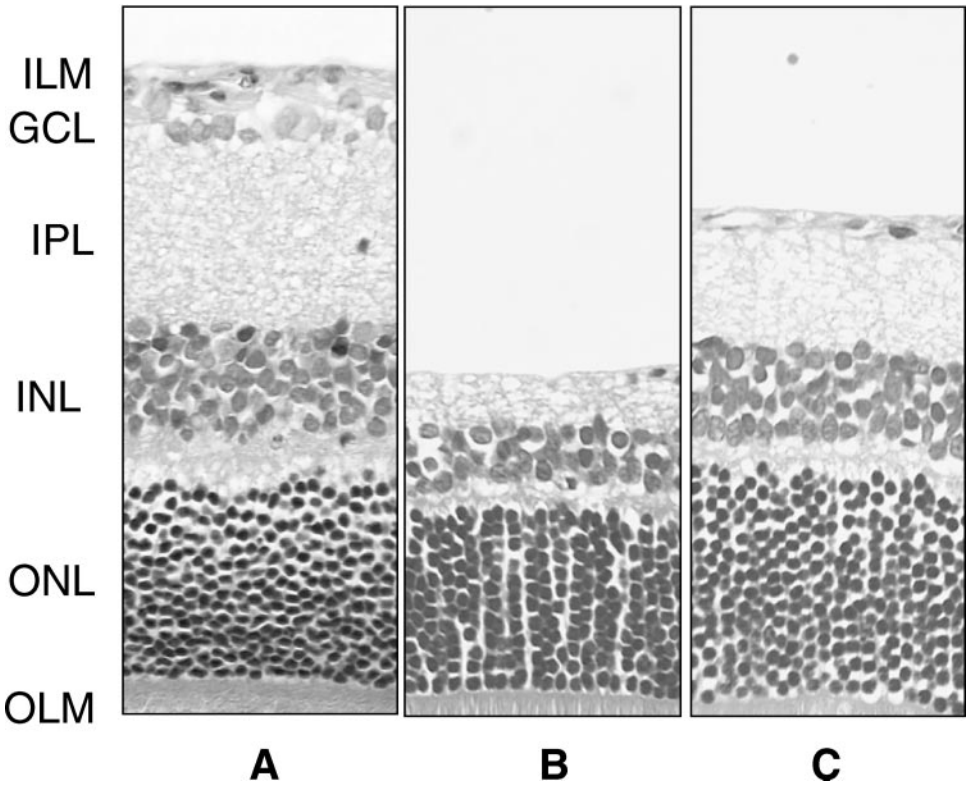


FIGURE 9. Inhibitory effect of AT-III on ischemia-induced retinal damage at 14 days after reperfusion: (**A**) control, (**B**) vehicle-treated retina, (**C**) AT-III–treated retina. Histologic examination showed a reduction in retinal thickness of the rats with induced ischemia. The protective effect of AT-III was most apparent in the inner retina. Original magnification, ×400.

ischemia.[29] Leukocytes that infiltrate postischemic tissues have been implicated as key mediators of ischemia–reperfusion injury, because they generate oxidants and release proteases.[28,41] Leukocyte infiltration from the mainstream circulation into postischemic tissue is mediated through a multistep process.[42–44] In the first step, leukocytes are tethered and roll along the vascular endothelial surface. This phenomenon is mediated primarily by endothelial P-selectin and carbohydrate ligands of leukocytes.[45,46] Meanwhile, some rolling leukocytes make firm adhesion to the vascular endothelium through $\beta 2$ integrins and ICAM-1, which leads to emigration from the vasculature.[47–49] In the postischemic retina, Tsujikawa et al.[50] have recently shown that P-selectin and ICAM-1 play a role in recruitment of leukocytes during reperfusion, and that inhibition of these adhesion molecules markedly decreases retinal damage subsequent to transient ischemia.

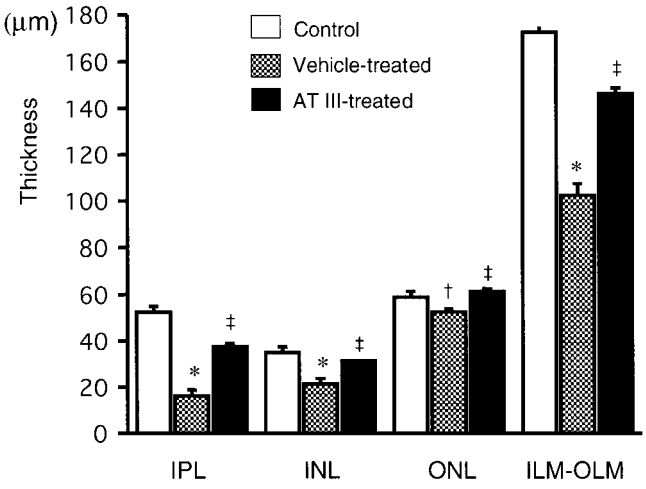


FIGURE 10. Thickness of different retinal layers in postischemic eyes at 14 days after reperfusion. Data are expressed as the mean ± SEM. $^*P < 0.01$ and $\dagger P < 0.05$ compared with control rats. $\ddagger P < 0.05$ compared with data in vehicle-treated rats.

In the study described herein, we showed that administration of AT-III just after the induction of retinal ischemia inhibited the accumulation of leukocytes after reperfusion. The maximum number of rolling and accumulating leukocytes was reduced by 56% and 47%, respectively, with treatment by AT-III. In postischemic liver, Harada et al.[30] similarly demonstrated that leukocyte accumulation after transient ischemia was reduced with treatment by AT-III. Recently, Ostrovsky et al.[25] reported that AT-III can inhibit leukocyte rolling and adhesion in postischemic feline mesentery. Because leukocyte rolling is the first and an indispensable step for accumulation, the inhibitory effect of AT-III on accumulation of leukocytes would be derived from the suppression of leukocyte rolling in postischemic retina. In their report, thrombin-induced rolling, but not histamine-induced rolling, could be rapidly reversed with treatment by AT-III. AT-III may exert its inhibitory effects on leukocyte-endothelial cell interactions in postischemic retina, not by blockage of adhesion molecules expressed on leukocytes or vascular endothelium, but rather by blockage of thrombin.

Recent evidence suggests that platelets also play a major role in the pathogenesis of ischemia–reperfusion injury.[5,6] This is based on evidence that depletion of circulating platelets reduces tissue damage after ischemia–reperfusion.[7–9] It is well known that platelets cause postischemic tissue damage through formation of thrombi.[51] However, other investigators have reported that platelets that accumulate in ischemic regions release oxidants and inflammatory mediators such as serotonin, leukotrienes, thromboxane-A_2, and platelet-derived growth factor, all of which can cause tissue damage.[10–14] Moreover, platelets reportedly recruit leukocytes to ischemic tissues through the expression of adhesion molecules on their surfaces or by release of cytokines.[15–19] Under physiologic conditions, platelets normally circulate without making inter-

actions with vascular endothelium. However, activated endothelial cells show interactions with platelets flowing in the marginal blood flow. In our previous report,[27] many platelets were observed rolling and adhering along the major retinal veins after transient retinal ischemia. We also reported that these interactions are mediated through expression of P-selectin on the retinal endothelial cells in postischemic retina, whereas P-selectin expressed on the platelets plays a minor role in these interactions.

The present study showed that administration of AT-III reduced substantially platelet–endothelial cell interaction in postischemic retina. The maximum number of rolling and adhering platelets was reduced by 69% and 46%, respectively, at 12 hours after reperfusion. Platelets that accumulate in vascular beds through endothelial P-selectin reportedly play a major role in the recruitment of leukocytes to inflammatory tissues. Several studies have demonstrated in vitro that platelet-immobilized collagen can support leukocyte rolling through platelet P-selectin.[15,52,53] In an in vivo experiment using intravital microscopy, Carvalho-Tavares et al.[54] Showed that depletion of platelets by antibodies against platelets markedly attenuates leukocyte rolling and adherence to endothelium that is activated by tumor necrosis factor-α. They reported that platelets appear to act as a bridge in linking some leukocytes to endothelium. In the present study, inhibition of the interactions between platelets and endothelial cells by AT-III may result in a stronger inhibitory effect on leukocyte–endothelial cell interactions.

AT-III inhibits thrombin directly through the formation of a stable stoichiometric covalent complex. In the present study, administration of AT-III reduced the expression of P-selectin in the retina after transient retinal ischemia. Histologic experiments showed that AT-III treatment succeeded in reducing postischemic retinal damage, especially inner retinal damage. This evidence suggests that AT-III reduces postischemic retinal damage by suppressing expression of these adhesion molecules on vascular endothelial cells, which results in the suppression of leukocyte and platelet accumulation in postischemic retina and, thus, reduces subsequent neural damage. However, Harada et al.[30] reported that AT-III may prevent ischemia–reperfusion-induced hepatic injury by increasing hepatic levels of prostacyclin (prostaglandin I_2 [PGI_2]) through the interaction of AT-III with cell-surface glycosaminoglycans. Because PGI_2 has been shown to inhibit platelet- and leukocyte-endothelial cell interactions, PGI_2 may also be involved in the neuroprotective effects of AT-III on the retinal ischemia–reperfusion injury.

In conclusion, both leukocytes and platelets were recruited to postischemic retina by their interactions with venous endothelial cells through adhesion molecules expressed on the damaged endothelial cells. These blood cells cause further tissue injury during reperfusion. The present study showed an inhibitory effect of AT-III on both leukocyte and platelet recruitment to postischemic retina. AT-III exists physiologically in plasma and is widely used in humans without any reported serious side effects. Therefore, AT-III may be effective in prevention and/or treatment of retinal ischemia–reperfusion injury.

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