Thrombin Inhibitor Reduces Leukocyte–Endothelial Cell Interactions and Vascular Leakage after Scatter Laser Photocoagulation

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Purpose. Macular edema is one of the most serious adverse effects after retinal scatter laser photocoagulation. It has been suggested that the inflammatory reaction after photocoagulation may be involved in the pathogenesis of macular edema. This study was designed to evaluate quantitatively the inhibitory effects of argatroban, a direct thrombin inhibitor, on leukocyte–endothelial cell interactions and vascular permeability after scatter laser photocoagulation.

Methods. Argon laser photocoagulation was performed in one half of the retina in pigmented male rats ($n = 114$). Argatroban was administered just before scatter laser photocoagulation. In the other half of the retina, AO leukocyte fluorography was used to evaluate in vivo leukocyte rolling along the retinal vein and accumulation in the retinal capillary bed. The expressions of P-selectin and intercellular adhesion molecule (ICAM)-1 were evaluated by reverse transcription–polymerase chain reaction. Retinal vessel permeability was quantified by using fluorescein isothiocyanate (FITC)-conjugated dextran.

Results. Scatter laser photocoagulation caused significant inflammatory leukocyte–endothelial cell interactions in the nonphotocoagulated half of the retina. Treatment with argatroban suppressed leukocyte–endothelial cell interactions. The maximum number of rolling and accumulating leukocytes was reduced by 46.6% ($P < 0.01$) and 51.4% ($P < 0.01$), respectively. The expression of P-selectin and ICAM-1 mRNA was suppressed significantly in the argatroban-treated retinas ($P < 0.05$). Retinal vascular permeability was also suppressed significantly ($P < 0.05$).

Conclusions. Argatroban suppressed leukocyte–endothelial cell interactions and blood–retinal barrier breakdown after scatter laser photocoagulation, suggesting that argatroban prevents postlaser macular edema. (*Invest Ophthalmol Vis Sci.* 2005;46:2561–2566) DOI:10.1167/iovs.04-1102

Scatter laser photocoagulation is widely used for the treatment of various retinal diseases, such as diabetic retinopathy, retinal vein occlusion, and retinopathy of prematurity. Despite the significant suppressive effect against neovascularization, macular edema is recognized as a potential side effect of scatter photocoagulation, resulting in transient or persistent visual disturbance.[1] Although it is serious and occurs frequently, however, the exact mechanisms of postlaser macular edema are not yet clear. The formation of edema after scatter laser photocoagulation usually occurs inside the vascular arcade, especially in the macula, whereas photocoagulation is performed outside the arcade vessels. Many recent reports have shown that treatment with scatter photocoagulation results in an increase in foveal thickness.[2,3] A pathogenic mechanism for the development of macular edema has been suggested to be the postlaser inflammatory reaction in the retina.[1]

We have developed a method of acridine orange (AO) leukocyte fluorography that allows us to visualize leukocytes and to evaluate quantitatively their behavior in the retinal microcirculation in vivo.[4,5] Using this method, we demonstrated impaired leukocyte flow in the untreated portion of the retina after partial scatter laser photocoagulation.[6] Moreover, we showed that inflammatory leukocyte–endothelial interactions were present in both the photocoagulated and nonphotocoagulated halves of the retina and demonstrated a significant increase of vascular permeability in the nonphotocoagulated portions of the retina 7 days after photocoagulation.[7] We hypothesized that leukocyte recruitment in the nonphotocoagulated posterior pole plays a role in the pathogenesis of macular edema after scatter laser photocoagulation.

When endothelial cells are activated, they express adhesion molecules that lead to leukocyte–endothelial cell interactions through a multistep process.[8,9] Initially, leukocytes interact with P-selectin, which is expressed on endothelial cells, and begin rolling along vessel walls. The leukocytes then interact with intracellular adhesion molecule (ICAM)-1, adhere to endothelial cells, and migrate out of the vessels. In this adhesion cascade, leukocytes are activated, finally injure the tissue, and may be responsible for increased microvascular permeability under inflammatory conditions.[10,11]

Recently, some investigators have shown that anticoagulant therapy attenuates inflammatory reactions, such as leukocyte–endothelial cell interactions or fibrin formation.[12–15] Argatroban, a selective thrombin inhibitor, is clinically used in vascular vessel-occlusive diseases[16,17] because it binds directly to the active site of thrombin and inhibits its function.[18,19] With the use of argatroban, prothrombin time (PT), showing the extrinsic coagulation pathway, and activated partial thromboplastin time (APTT), showing the intrinsic coagulation pathway, have been prolonged, and platelet aggregation has been shown to be inhibited.[19,20]

The purpose of this study was to evaluate quantitatively the inhibitory effects of argatroban on leukocyte–endothelial cell interactions and retinal vascular permeability in the nonphotocoagulated portions of the retina, after partial scatter laser photocoagulation.

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Supported by a grant from the Japan National Society for the Prevention of Blindness and a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science.

Submitted for publication September 16, 2004; revised February 14, 2005; accepted March 3, 2005.

Disclosure: **K. Musashi**, None; **J. Kiryu**, None; **K. Miyamoto**, None; **S. Miyahara**, None; **H. Katsuta**, None; **H. Tamura**, None; **F. Hirose**, None; **N. Yoshimura**, None

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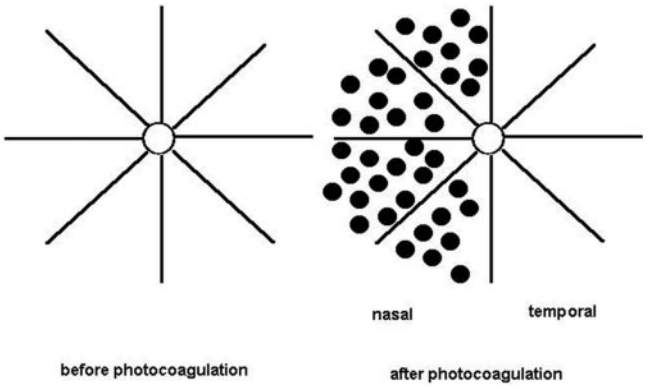

FIGURE 1. Representation of scatter photocoagulation to the rat fundus. *Central circle*: optic disc; *black dots*: laser burns. Photocoagulation was delivered to half the retina; the other was untreated.

METHODS

Animal Model

Pigmented male Long-Evans rats (200–250 g; $n = 114$; Kiwa Laboratory Animals Co., Ltd., Wakayama, Japan) were anesthetized with xylazine hydrochloride (4 mg/kg) and ketamine hydrochloride (10 mg/kg). The pupils were dilated with 0.5% tropicamide and 2.5% phenylephrine hydrochloride. A contact lens was placed on the cornea to maintain transparency. Argon laser photocoagulation was delivered through a slit lamp biomicroscope equipped with a 90-D fundus lens. A total of 400 spots of scatter laser burns were placed in half of the retina, and the other half was left untreated. The laser treatment was applied at the following settings: spot size, 100 μm; duration, 0.05 second; and power, 70 mW.[6,7] The spots were placed 1 or 1.5 spot diameters peripherally from the optic disc. Only one eye was treated in each animal (Fig. 1).

Argatroban (Mitsubishi Pharma, Osaka, Japan; and Daiichi Pharmaceutical, Montvale, NJ) was dissolved in 0.9% NaCl solution containing HCl to prepare concentrations of 30 mg/mL. The vehicle was 0.9% NaCl solution containing HCl. The argatroban solution or vehicle was infused with the use of an osmotic pump (10 μL/h) that was implanted intraperitoneally just before retinal scatter laser photocoagulation was performed.

Peripheral blood specimens were collected to count the number of leukocytes and platelets with the use of a hematology system (Erma; Bayer Diagnostics, Munich, Germany) and to measure the PT and APTT at various time points. All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

AO Leukocyte Fluorography

At 12, 18, 24, 36, and 48 hours after 400 spots of photocoagulation were applied, AO leukocyte fluorography was performed. Six rats in both the argatroban-treated and vehicle-treated groups were used at each time point. Another six nonsurgical rats served as the control. To evaluate leukocyte–endothelial cell interactions after retinal scatter laser photocoagulation, we used AO leukocyte fluorography, which has been described in detail elsewhere.[4,5] In brief, a scanning laser ophthalmoscope (SLO; Rodenstock Instruments, Munich, Germany), coupled with a computer-assisted image-analysis system, made continuous, high-resolution images of the fundus stained by metachromatic fluorochrome AO (Wako Pure Chemicals, Osaka, Japan). For further analysis, the obtained images were recorded on an S-VHS videotape at a rate of 30 frames/s. Immediately before AO leukocyte fluorography, the rats were anesthetized, and the pupils were dilated. A contact lens was used to retain corneal clarity throughout the experiment. Arterial blood pressure was monitored with a blood pressure analyzer (IITC Life Sciences, Woodland Hills, CA). AO (0.1% solution in saline) was injected continuously through the tail vein catheter for 1 minute at a rate of 1 mL/min. 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 from the number of cells per minute crossing a fixed area of the vessel at a distance of two disc diameters from the optic disc center. At 30 minutes after the injection of AO, the fundus was observed again, to evaluate leukocyte accumulation in the retinal microcirculation. The number of fluorescent dots was counted in 1-mm^2 areas of the nonphotocoagulated portions of the retina at a distance of 1 disc diameter from the edge of the optic disc.

Semiquantification of P-selectin and ICAM-1 Gene Expression

After 12 and 24 hours of photocoagulation, one eye of each of the six rats in the argatroban-treated, vehicle-treated, and nonsurgical control groups was enucleated. Total RNA was isolated from the retina according to the acid guanidinium-thiocyanate-phenol-chloroform extraction method.[21] The extracted RNA was quantified, and then 2 μg was used to make cDNA, with a kit (Omniscript reverse transcriptase; Qiagen, Hilden, Germany). Polymerase chain reaction was performed by the method of Saiki et al.[22] with slight modification. The following conditions were used: denaturation at 94°C for 1 minute, annealing at 62°C for 1 minute, and polymerization at 72°C for 1 minute. The reaction was performed for 31 cycles. The primers were TGCTTGGCTACTGGACACTG (sense) and GGTGTCGACAGGACATTGTG (antisense) for P-selectin, AGCCTCAGGCCTAAGAGGAC (sense) and AGGGGTCCCAGAGAGGTCTA (antisense) for ICAM-I, and GGCATCCTGACCCTGAAGTA (sense) and GCCATCTCTTGCTCGAAGTC (antisense) for β-actin. Nucleotide sequencing and restriction pattern analysis confirmed that polymerase chain reaction products were derived from the target cDNA sequences.

Measurement of Retinal Vessel Permeability with FITC-Dextran

Retinal blood vessel leakage in the rat was quantitated at 168 hours after 400 spots of photocoagulation, as previously described in detail.[23] After deep anesthesia with xylazine hydrochloride and ketamine hydrochloride, fluorescein isothiocyanate (FITC)–conjugated dextran (4.4 kDa, 50 mg/mL in PBS, 50 mg/kg body weight; Sigma-Aldrich, Tokyo, Japan) was injected intravenously. After 10 minutes, the chest cavity was opened, and a 14-gauge perfusion cannula was introduced into the aorta. A blood sample was collected immediately before perfusion. After drainage from the right atrium was achieved, each rat was perfused with PBS (500 mL/kg body weight), to clear the remaining intravascular dextran. The blood sample was centrifuged at 7000 rpm for 20 minutes at 4°C, and the supernatant was diluted at 1:1000. Immediately after perfusion, one eye from each of six rats in the argatroban-treated, vehicle-treated, and nonsurgical control groups was enucleated. The nonphotocoagulated portions of the retina were carefully removed, weighed, and homogenized to extract the FITC-dextran in 0.4 mL of water. The extract was processed through a 30,000-molecular-weight filter (Ultrafree-MC; Millipore, Bedford, MA) at 7000 rpm for 90 minutes at 4°C. The fluorescence in each 300-μL sample was measured (excitation, 485 nm; emission, 538 nm) with a spectrofluorometer (SPECTRAmax GEMINI XS; Molecular Devices, Sunnyvale, CA) with water as a blank. Corrections were made by subtracting the autofluorescence of retinal tissue from rats without FITC-dextran injection. The amount of FITC-dextran in each retina was calculated from a standard curve of FITC-dextran in water. For normalization, the retinal FITC-dextran amount was divided by retinal weight and by the concentration of FITC-dextran in the plasma.

Statistical Analysis

All data are presented as the mean ± SD. Student's *t*-test was used to compare two groups. ANOVA was used to compare three or more conditions, with post hoc comparisons tested with the Fisher pro-

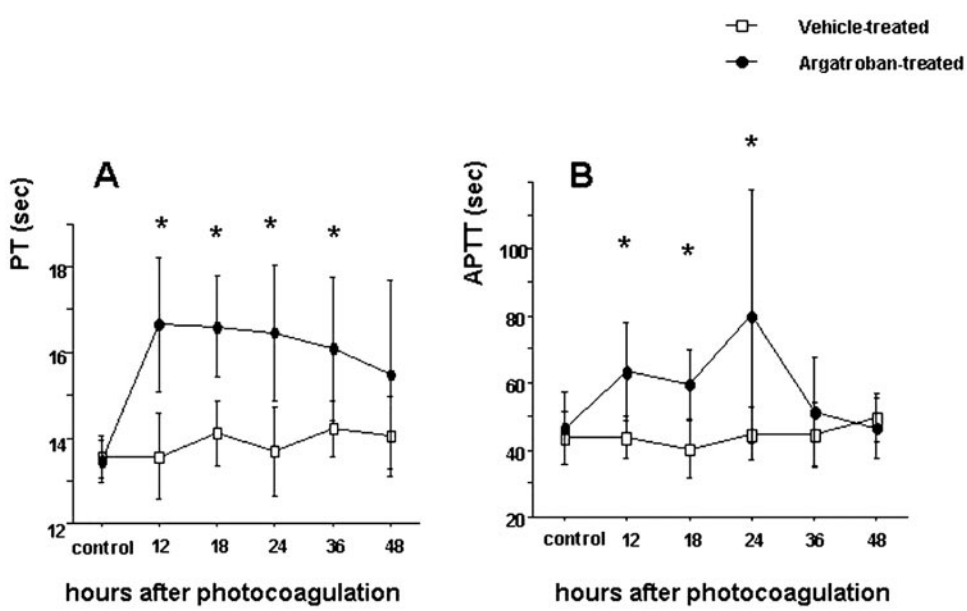

FIGURE 2. Time course of (**A**) PT and (**B**) APTT after retinal scatter laser photocoagulation. Data are the mean ± SD. $^*P < 0.05$ compared with vehicle-treated rats.

tected least-significant difference procedure. Differences were considered statistically significant at $P < 0.05$.

RESULTS

PT and APTT

Both PT and APTT were prolonged after scatter laser photocoagulation in the argatroban-treated group compared with the vehicle-treated group (Fig. 2). APTT was significantly prolonged with treatment by argatroban at 12, 18, and 24 ($P < 0.05$) hours after scatter laser photocoagulation. PT was also significantly prolonged by treatment with argatroban at 12, 18, 24, and 36 ($P < 0.05$) hours after photocoagulation.

Physiologic Data

At various time points after scatter laser photocoagulation, physiologic variables, such as the number of leukocytes and platelets, systolic blood pressure, and heart rate were measured. There were no significant differences between the argatroban- and vehicle-treated groups in any of the physiologic parameters studied.

Leukocyte Rolling

Immediately after AO was infused intravenously, only leukocytes were stained among the circulating blood cells. No rolling leukocytes were observed in the nonsurgical control group. In the laser-treated rats, some leukocytes were observed slowly rolling along major retinal veins but not along any major retinal arteries. In the vehicle-treated group, the flux of rolling leukocytes increased substantially and peaked at 18 hours after scatter laser photocoagulation (12.4 ± 3.1 cells/min). In the argatroban-treated group, leukocyte rolling was significantly inhibited compared with that in the vehicle-treated group ($P < 0.001$; Fig. 3A). The number of rolling leukocytes in the argatroban-treated group was reduced to 46.6% of that in the vehicle-treated group at 18 hours after photocoagulation ($P < 0.0051$).

Leukocyte Accumulation

Figure 3B indicates a change in the number of leukocytes accumulating in the retinal microcirculation in the argatroban- and vehicle-treated groups. Few leukocytes were found in the nonsurgical control retinas. In the vehicle-treated group, accumulating leukocytes began to increase with time after scatter laser photocoagulation and peaked at 24 hours after photocoagulation (59.3 ± 16.1 cells/mm^2). The number of accumulating leukocytes was significantly decreased in the argatroban-treated group compared with the vehicle-treated group ($P < 0.001$). With argatroban treatment, the number of accumulating leukocytes was reduced to 51.4% at 24 hours after scatter laser photocoagulation ($P = 0.0055$).

Figure 3C shows accumulated leukocytes after AO injection. A small number of leukocytes was found in control rats. An increasing number of leukocytes accumulated at 24 hours after photocoagulation in the vehicle-treated group. A significant reduction of leukocyte accumulation was seen in the argatroban-treated group.

P-selectin and ICAM-I Gene Expression

The levels of gene expression are shown as a ratio to the average values of nonsurgical control rats (Fig. 4). ICAM-I mRNA expression was upregulated in the vehicle-treated group ($P = 0.0006$) and was significantly suppressed in the argatroban-treated group at 24 hours after retinal scatter laser photocoagulation ($P = 0.016$). P-selectin mRNA expression was also upregulated in the vehicle-treated group ($P = 0.0090$) and was significantly suppressed in the argatroban-treated group ($P = 0.046$) at 12 hours after photocoagulation.

Retinal Vessel Permeability in Nonphotocoagulated Retina

The levels of vascular permeability are shown as a ratio to the average values of nonsurgical control rats (Fig. 5). The vascular permeability was upregulated in the vehicle-treated group ($P = 0.0048$) and was significantly suppressed in the argatroban-treated group ($P = 0.033$) at 168 hours after photocoagulation. There were no significant differences between the argatroban-treated and nonsurgical control group ($P = 0.36$).

DISCUSSION

Our results demonstrated that argatroban, a direct thrombin inhibitor, could suppress leukocyte–endothelial cell interactions in the postlaser rat retina. The mRNA expressions of

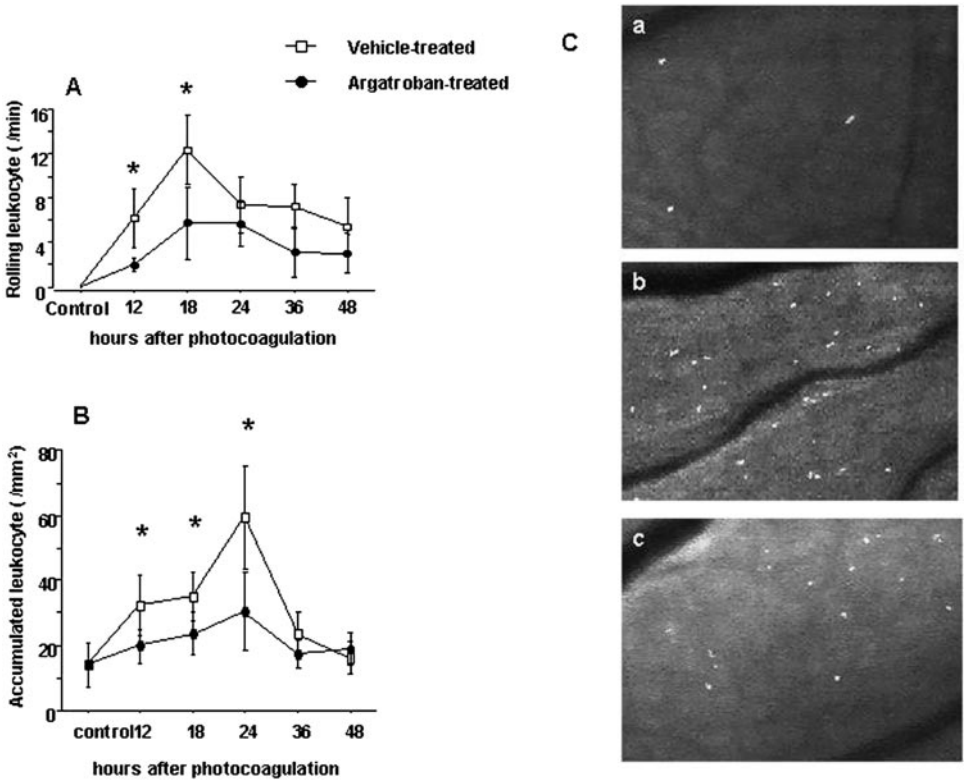

FIGURE 3. Time course of the number of (**A**) rolling leukocytes and (**B**) accumulating leukocytes after retinal scatter laser photocoagulation. Data are mean ± SD. $P < 0.05$ compared with vehicle-treated rats. (**C**) Leukocyte accumulation after AO injection. (**Ca**) Nonsurgical control group; (**Cb**) vehicle-treated group; and (**Cc**) argatroban-treated group.

P-selectin and ICAM-I were also suppressed significantly in the argatroban-treated retina. On the basis of these findings, we suggest that argatroban may attenuate blood–retinal barrier breakdown after retinal scatter laser photocoagulation, not only by inhibiting coagulation but also by inhibiting inflammatory reactions mediated by accumulated leukocytes and platelets.

We previously showed that inflammatory leukocyte–endothelial interactions were observed in both the photocoagulated and the nonphotocoagulated halves of rat retina and demonstrated a significant increase of vascular permeability in the nonphotocoagulated portions.[7] An investigation using the fluorophotometric technique developed by Taguchi et al.[24] has demonstrated that hydrogen peroxide diffused into the vitreous after retinal scatter photocoagulation. Hydrogen peroxide was found to induce upregulation of adhesion molecules on the vascular endothelium and subsequent leukocyte–endothelial interactions.[25] Accordingly, scatter photocoagulation may cause inflammatory leukocyte behavior, such as rolling and accumulation in the nonphotocoagulated retina through oxidative stress-induced upregulation of adhesion molecules on the vascular endothelium.

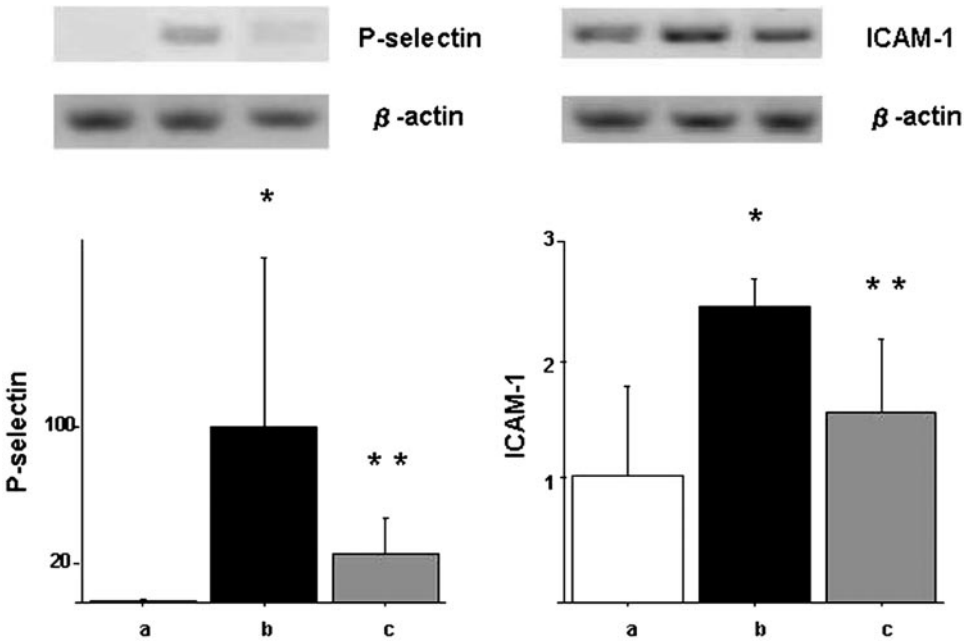

FIGURE 4. Gene expression of P-selectin and ICAM-I after retinal scatter laser photocoagulation. a, nonsurgical control group; b, vehicle-treated group; and c, argatroban-treated group. Levels of gene expression are shown as a ratio of the average levels in control rats. Data are the mean ± SD. $^{*}P < 0.05$ compared with control rats; $^{**}P < 0.05$ compared with vehicle-treated rats.

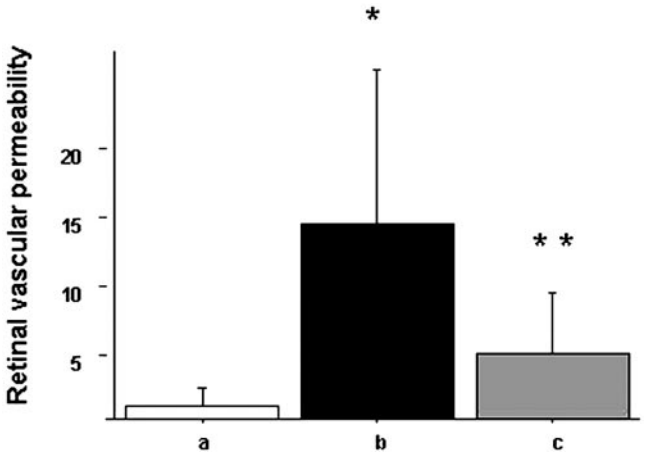

FIGURE 5. Blood–retinal barrier breakdown after retinal scatter laser photocoagulation. a, nonsurgical control group; b, vehicle-treated group; and c, argatroban-treated group. Levels of breakdown are shown as the ratio to the average level in control rats (a, 1 ± 1.66; b, 16.4 ± 12.9; and c, 5.44 ± 5.06). Data are the mean ± SD. $^{*}P < 0.05$ compared with control rats; $^{**}P < 0.05$ compared with vehicle-treated rats.

Recent evidence suggests that leukocytes and platelets play a critical role in disrupting the microvascular barrier in various organs.[26–28] These reports have suggested that leukocyte–endothelial contact, such as leukocyte adhesion on the vascular endothelium and subsequent leukocyte extravasation, may be responsible for increased microvascular permeability under inflammatory conditions. Using AO leukocyte fluorography, we have demonstrated in vivo that platelet depletion suppresses leukocyte rolling and subsequent accumulation in postischemic tissues.[26] Platelets adhering to vascular endothelium contribute to the recruitment of leukocytes through the expression of P-selectin on their surfaces and participate in leukocyte-dependent tissue injury.[26] Moreover, thrombin activates platelets and causes them to produce inflammatory mediators, such as serotonin, leukotrienes, thromboxane A2, monocyte chemotactic protein-3, and platelet-derived growth factor.[29,30] Some investigators have shown that anticoagulant therapy attenuates the inflammatory reactions, such as leukocyte–endothelial cell interactions, or fibrin formation.[12–15] We demonstrated previously that argatroban could also suppress leukocyte- and platelet–endothelial cell interactions in the postischemic rat retina and that the expression of mRNA of P-selectin and ICAM-I was suppressed significantly in the argatroban-treated retina.[15] We hypothesized that argatroban, a selective thrombin inhibitor, has a potential effect on the blood–retinal barrier breakdown by inhibition of the aggregation of platelets.

Wilson et al.[31] showed the gene expression in the mouse retina 3 days after argon laser photocoagulation, as determined by microarray analysis. In their study, both genes, P-selectin and ICAM-I, were not the upregulated ones. Conversely, our results showed mRNA upregulation of these adhesion molecules in the nonphotocoagulated portions of the retina. The time course of postlaser reaction may account for these conflicting results, as reported by Wilson et al. and us. In the acute phase, the mRNA expressions of these adhesion molecules were upregulated after scatter laser photocoagulation. Then, argatroban inhibited the mRNA expressions of these adhesion molecules. The suppressed leukocyte–endothelial cell interactions in the postlaser retina of argatroban-treated rats were associated with the suppressed expressions of these adhesion molecules.

In conclusion, we have demonstrated that argatroban can attenuate leukocyte–endothelial cell interactions and blood–retinal barrier breakdown in the nonphotocoagulated portions of the retina after partial scatter laser photocoagulation. These results suggest that argatroban attenuates the development of macular edema by inhibiting inflammatory reactions mediated by leukocytes and platelets. Argatroban may have a therapeutic effect on macular edema after photocoagulation.

References

1. McDonald HR, Schatz H. Macular edema following panretinal photocoagulation. *Retina*. 1985;5:5–10.
2. Tsujikawa A, Kiryu J, Dong J, et al. Quantitative analysis of diabetic macular edema after scatter laser photocoagulation with the scanning retinal thickness analyzer. *Retina*. 1999;19:59–64.
3. Shimura M, Yasuda K, Tamai M, et al. Quantifying alterations of macular thickness before and after panretinal photocoagulation in patients with severe diabetic retinopathy and good vision. *Ophthalmology*. 2003;110:2386–2394.
4. Nishiwaki H, Ogura Y, Honda Y, et al. Quantitative evaluation of leukocyte dynamics in retinal microcirculation. *Invest Ophthalmol Vis Sci*. 1995;36:123–130.
5. Nishiwaki H, Ogura Y, et al. Visualization and quantitative analysis of leukocyte dynamics in retinal microcirculation of rats. *Invest Ophthalmol Vis Sci*. 1996;37:1341–1347.
6. Hiroshiba N, Ogura Y, Honda Y, et al. Alterations of retinal microcirculation in response to scatter photocoagulation. *Invest Ophthalmol Vis Sci*. 1998;39:769–776.
7. Nonaka A, Kiryu J, Honda Y, et al. Inflammatory response after scatter laser photocoagulation in nonphotocoagulated retina. *Invest Ophthalmol Vis Sci*. 2002;43:1204–1209.
8. Sugama Y, Tiruppathi C, Malik AB, et al. Thrombin-induced expression of endothelial P-selectin and intercellular adhesion molecule-1: a mechanism for stabilizing neutrophil adhesion. *J Cell Biol*. 1992;119:935–944.
9. Sluiter W, Pietersma A, Koster JF, et al. Leukocyte adhesion molecules on the vascular endothelium: their role in the pathogenesis of cardiovascular disease and the mechanisms underlying their expression. *J Cardiovasc Pharmacol*. 1993;22(suppl 4):S37–S44.
10. Matsuo Y, Kihara T, Kogure K, et al. Role of neutrophils in radical production during ischemia and reperfusion of the rat brain: effect of neutrophil depletion on extracellular ascorbyl radical formation. *J Cereb Blood Flow Metab*. 1995;15:941–947.
11. Ghezzi P, Dinarello CA, White CW, et al. Hypoxia increases production of interleukin-1 and tumor necrosis factor by human mononuclear cells. *Cytokine.* 199;3:189–194.
12. Tyrrel DJ, Horne AP, Page CP, et al. Heparin in inflammation: potential therapeutic applications beyond anticoagulation. *Adv Pharmacol*. 1999;46:151–208.
13. Yamashiro K, Kiryu J, Honda Y, et al. Inhibitory effects of antithrombin III against leukocyte rolling and infiltration during endotoxin induced uveitis in rats. *Invest Ophthalmol Vis Sci*. 2001;42:1553–1560.
14. Maeno T, Tano Y, Takenaka H, et al. Argatroban inhibits intraocular fibrin formation after vitrectomy in rabbits. *Arch Ophthalmol*. 2000;118:1401–1405.
15. Miyahara S, Kiryu J, Honda Y, et al. Argatroban attenuates leukocyte- and platelet-endothelial cell interactions after transient retinal ischemia. *Stroke*. 2003;34:2043–2049.
16. Gregory WA. Antithrombotic agents in cerebral ischemia. *Am J Cardiol*. 1995;75:34B–38B.
17. Kario K, Kodama K, Matsuo T, et al. Thrombin inhibition in the acute phase of ischaemic stroke using argatroban. *Blood Coagul Fibrinolysis*. 1995;6:423–427.
18. Okamoto S, Sugano M, Tamao Y, et al. Potent inhibition of thrombin by the newly synthesized arginine derivative no. 805: the importance of stereo-structure of its hydrophobic carboxamide portion. *Biochem Biophys Res Commun*. 1981;101:440–446.

19. Green D, Kohl H, Cohen I, et al. In vitro studies of a new synthetic thrombin inhibitor. *Thromb Res Suppl*. 1985;37:145–153.
20. Imiya M, Matsuo T. Inhibition of collagen-induced platelet aggregation by argatroban in patients with acute cerebral infarction. *Thromb Res*. 1997;88:245–250.
21. Chomczynski P, Sacchi N. Single method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem*. 1987;162:156–159.
22. Saiki RK, Gelfand DH, Erlich HA, et al. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*. 1988;239:487–491.
23. Yamashiro K, Tsujikawa A, Honda Y, et al. Platelets accumulated in the diabetic retinal vasculature following endothelial death and suppress blood-retinal barrier breakdown. *Am J Pathol*. 2003;163:253–259.
24. Taguchi H, Ogura Y, Honda Y, et al. Fluorophotometric detection of intravitreal peroxides after panretinal laser photocoagulation. *Invest Ophthalmol Vis Sci*. 1998;39:358–363.
25. Gaboury JP, Anderson DC, Kubes P. Molecular mechanisms involved in superoxide-induced leukocyte–endothelial cell interactions in vivo. *Am J Physiol*. 1994;266:H637–H642.
26. Nishijima K, Kiryu J, Honda Y, et al. Platelets adhering to the vascular wall mediate postischemic leukocyte–endothelial cell interactions in retinal microcirculation. *Invest Ophthalmol Vis Sci*. 2004;45:977–984.
27. Celi A, Lorenzet R, Furie BC, et al. Platelet-leukocyte–endothelial cell interaction on the blood vessel wall. *Semin Hematol*. 1997;34:327–335.
28. Bolton SJ, Anthony DC, Perry VH. Loss of the tight junction proteins occludin and zonula occludens-1 from cerebral vascular endothelium during neutrophil-induced blood-brain barrier breakdown in vivo. *Neuroscience*. 1998;86:1245–1257.
29. Piccardoni P, Walz A, Cerletti C, et al. Thrombin activated human platelets release two NAP 2 variants that stimulate polymorphonuclear leukocytes. *Thromb Haemost*. 1996;76:780–785.
30. Kuijper PHM, Torres HIG, Koenderman L, et al. Platelet-dependent primary hemostasis promotes selectin- and integrin-mediated neutrophil adhesion to damaged endothelium under flow conditions. *Blood*. 1996;87:3271–3281.
31. Wison A, Rakoczy P, Hobbs B, et al. Argon laser photocoagulation-induced modification of gene expression in the retina. *Invest Ophthalmol Vis Sci*. 2003;44:1426–1414.