Argatroban Attenuates Leukocyte– and Platelet–Endothelial Cell Interactions After Transient Retinal Ischemia

Shinsuke Miyahara, MD; Junichi Kiryu, MD; Akitaka Tsujikawa, MD; Hideto Katsuta, MD; Kazuaki Nishijima, MD; Kazuaki Miyamoto, MD; Kenji Yamashiro, MD; Atsushi Nonaka, MD; Yoshihito Honda, MD

Background and Purpose—Argatroban, a direct thrombin inhibitor, has been shown to reduce neural injury after transient cerebral ischemia. It has also been reported that this neuroprotective effect results from an anticoagulant function. This study was designed to evaluate quantitatively the inhibitory effects of argatroban on leukocyte– and platelet–endothelial cell interactions after transient retinal ischemia.

Methods—Retinal ischemia was induced for 60 minutes in male Long-Evans rats by temporary ligation of the optic sheath (n = 342). Argatroban was administered just after induction of ischemia. Leukocyte and platelet behavior in the retinal microcirculation was then evaluated in vivo with scanning laser ophthalmoscopy. The expression of P-selectin and intracellular adhesion molecule-1 (ICAM-1) was evaluated by reverse transcription–polymerase chain reaction. After 10 days of reperfusion, ischemia-induced retinal damage was evaluated histologically.

Results—Treatment with argatroban suppressed leukocyte–endothelial cell interactions; the maximum numbers of rolling and accumulated leukocytes were reduced by 90.1% (P < 0.05) and 58.7% (P < 0.05), respectively, at 12 hours after reperfusion. Treatment with argatroban also suppressed platelet–endothelial cell interactions; the maximum numbers of rolling and adhering platelets were reduced by 91.8% (P < 0.01) and 78.9% (P < 0.01), respectively, at 12 hours after reperfusion. The expression of P-selectin and ICAM-1 mRNA was suppressed significantly in the argatroban-treated retinas (P < 0.01). Histologic examination demonstrated the protective effect of argatroban on ischemia-induced retinal damage (P < 0.01).

Conclusions—Argatroban treatment suppressed leukocyte– and platelet–endothelial cell interactions after transient retinal ischemia. This inhibitory effect on postischemic blood cell–endothelial cell interactions might partially contribute to its neuroprotective effects. (Stroke. 2003;34:2043-2049.)

Key Words: ischemia • leukocytes • platelets • retina • rheology

Argatroban, a selective thrombin inhibitor, is clinically used in vascular-vessel occlusive diseases because it binds directly to the active site of thrombin and inhibits its function. With the use of argatroban, thrombin time, prothrombin time (PT), and activated partial thromboplastin time (APTT) have been prolonged, and platelet aggregation has been shown to be inhibited. Recently, some investigators have reported that argatroban can attenuate damages after transient cerebral ischemia by inhibiting edema and improving cerebral blood flow.

Leukocytes play a major role in inflammatory injury after transient ischemia. When endothelial cells are activated by transient ischemia, they express adhesion molecules that lead to leukocyte–endothelial interactions through a multi-step process. Initially, leukocytes interact with P-selectin that is expressed on endothelial cells and begin rolling along vessel walls. The leukocytes then interact with intracellular adhesion molecule-1 (ICAM-1), adhere to endothelial cells, and migrate out of the vessels. In this adhesion cascade, leukocytes are activated and finally injure the tissue because of their inflammatory reactions. Because thrombin is thought to play a role in the induction of adhesion molecules after transient ischemia, argatroban might exert its neuroprotective effects by suppressing these adhesion molecules, resulting in the inhibition of leukocyte-mediated tissue damage after transient cerebral ischemia.

Moreover, several reports have suggested that platelets also play an important role in the pathogenesis of injury after transient ischemia. Platelets interact with activated endothelial cells and accumulate in the injured region. In vivo studies have shown that platelets can roll on activated endothelium by means of P-selectin that is expressed on the endothelial cells in the course of their accumulation in the inflamed tissue. In the injured region, platelets produce...
inflammatory mediators\textsuperscript{20,21} and recruit leukocytes to ischemic regions through the expression of adhesion molecules on their surfaces or by the production of cytokines.\textsuperscript{22,23} Argatroban prevents coagulation by inhibiting platelet–platelet interaction and fibrin formation, events that could otherwise lead to inhibition of postischemic thrombus formation.\textsuperscript{4,5} Argatroban might also prevent inflammation by inhibiting platelet–endothelial cell interactions.

We have developed in vivo methods to quantitatively evaluate leukocyte-\textsuperscript{10,24,25} and platelet-\textsuperscript{18,19} endothelial cell interactions in the rat retina. The optic media, which consists of the cornea, lens, vitreous, and retina, are so transparent that the retinal microcirculation can be observed noninvasively in vivo. The retina is part of the central nervous system, and the properties of endothelial cells and neural cells in the retina are similar to those in the cerebrum.\textsuperscript{26,27} Therefore, investigation of leukocyte and platelet dynamics in postischemic retina might be extrapolated to leukocyte or platelet involvement in postischemic brain injury. The purpose of this study was to evaluate quantitatively the inhibitory effects of argatroban on leukocyte– and platelet–endothelial cell interactions in vivo in postischemic retina and to study the therapeutic efficacy of argatroban on retinal injury after transient ischemia.

Materials and Methods

Animal Model

Induction of transient retinal ischemia was reported previously.\textsuperscript{10,25} Male pigmented Long-Evans rats (200 to 220 g; n = 542) (KIWA Laboratory Animals Co, Ltd) 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. After a lateral conjunctival peritomy and disinsertion of the lateral rectus muscle, the optic sheath of the right eye was exposed by blunt dissection, and a 6-0 nylon suture was passed around the optic sheath and tightened until blood flow ceased in all retinal vessels. The absence of perfusion for a 60-minute period was confirmed with the use of an operating microscope, after which the suture was removed.

Argatroban (obtained from Mitsubishi Pharma Corporation/Daiichi Pharmaceutical) 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. Argatroban solution or vehicle was infused by way of an osmotic pump (10 m\textsuperscript{3} L/h) that was implanted intraperitoneally immediately after ischemia induction. To investigate the dose dependence of argatroban, 2 additional doses of argatroban (3 and 0.3 mg/mL) were used.

Peripheral blood specimens were collected to count the number of leukocytes and platelets with use of a hematology analyzer (ERMA) and to measure the PT and APTT at various reperfusion points. All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Leukocyte–Endothelial Cell Interactions

To evaluate leukocyte–endothelial cell interactions after transient retinal ischemia, we used fluorescently labeled platelets, a technique that has been described in detail elsewhere.\textsuperscript{18,19} In brief, platelet samples were harvested from donor rats and stained with carboxyfluorescein diacetate succinimidyl ester (Molecular Probes). After each rat was anesthetized, 6\times 10\textsuperscript{5} fluorescently labeled platelets were infused into the tail vein catheter. Platelet behavior in the retinal microcirculation was then observed with an SLO and recorded for further analysis.

Platelet behavior in the retinal microcirculation was evaluated at 4, 12, 24, and 48 hours after reperfusion in both argatroban-treated and vehicle-treated groups. Six different rats were used at each time point. Rolling platelets were defined as those that moved at a velocity slower than that of free-flowing platelets. The number of rolling platelets in each major retinal vein was calculated for 1 minute at 2 disk diameters from the center of the optic disk. The total number of rolling platelets along all major veins was used as the number of rolling platelets in each rat. A platelet was defined as adherent to vascular endothelium if it remained stationary for >10 seconds. Adherent platelets were calculated as the total number of adherent platelets along all major retinal veins identified for 1 minute within a circle with a radius of 500 \textmu m from the center of the optic disk. All parameters were evaluated after a stabilization period of 5 minutes after the administration of platelets.

Semi-quantification of P-Selectin and ICAM-I Gene Expression

After 6 hours of reperfusion, 1 eye from each of 6 rats in the argatroban-treated, vehicle-treated, and nonoperated control groups was enucleated. Total RNA was isolated from the retina according to the acid guanidinium thiocyanate-phenol-chloroform extraction method.\textsuperscript{29} The extracted RNA was quantified, and then 2 \mu g was used to make cDNA with use of a kit (Omniscript reverse transcriptase, QIAGEN). Polymerase chain reaction was performed with the method of Saiki et al.,\textsuperscript{30} 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 TGCTTG-GCTACTGGAGACTG (sense) and GGTGTCGACAGGACATTTG (antisense) for P-selectin, AGCCCTAGGCTAAAGGAGAC (sense) and AGGGGTCCAGAGGAGTCTA (antisense) for ICAM-I, and GGATCCTGACCTGAAGTA (sense) and GC- CATCTCTTGCTGAAGTC (antisense) for \beta-actin. Nucleotide sequencing and restriction pattern analysis confirmed that polymerase chain reaction products were derived from the target cDNA sequences.
PT and APTT were prolonged after reperfusion in the argatroban-treated group compared with the vehicle-treated group (Figure 1). APTT was significantly prolonged with treatment by argatroban at 4 (P=0.011) and 12 (P=0.0088) hours after reperfusion; PT was also significantly prolonged by treatment with argatroban at 4 (P=0.004) and 12 (P=0.036) hours after reperfusion.

**Physiologic Data**

The Table indicates the changes in physiologic variables and diameters of the major retinal vessels at various time points after transient ischemia. There were no significant differences between the argatroban-treated 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 nonoperated control group. In the operated rats, some leukocytes were observed slowly rolling along major retinal veins but not along any major retinal arteries. In the vehicle-treated group, a few leukocytes were observed rolling along the venous walls at 4 hours after reperfusion. The flux of rolling leukocytes increased substantially and peaked at 12 hours after reperfusion (192.3±61.3 cells/min). In the argatroban-treated group, leukocyte rolling was significantly inhibited compared with that in the vehicle-treated group (P=0.015; Figure 2A). The number of rolling leukocytes in the argatroban-treated group was reduced to 9.9% of that in the vehicle-treated group at 12 hours after reperfusion (P=0.019).

**Leukocyte Accumulation**

Figure 2B indicates changes in the numbers of leukocytes accumulated in the retinal microcirculation in the argatroban-treated and vehicle-treated groups; few leukocytes could be found in the nonoperated control retinas. In the vehicle-treated group, accumulated leukocytes began to increase with time after reperfusion and peaked at 12 hours after reperfusion (934.6±196.0 cells/mm²). The number of accumulated leukocytes was significantly decreased in the argatroban-treated group compared with the vehicle-treated group (P=0.017). With argatroban treatment, the number of accumulated leukocytes was reduced to 41.3% at 12 hours after reperfusion (P=0.034).

**Platelet Rolling and Adhesion**

Immediately after labeled platelets were infused intravenously, fluorescent platelets were visibly circulating in the

---

**Histologic Evaluation**

After 10 days of reperfusion, 1 eye each from 6 rats in the argatroban-treated, vehicle-treated, and nonoperated control groups was obtained to evaluate the severity of retinal damage. These eyes were fixed in 1.48% formaldehyde and 1% glutaraldehyde in phosphate buffer and then in 3.7% formaldehyde. The eyes were then dehydrated, embedded in paraffin, sectioned with a microtome at 4-μm thickness, and stained with hematoxylin and eosin. Each section was cut along the horizontal meridian of the eye through the optic nerve head; sections were cut perpendicular to the retinal surface. Retinal sections were examined with an optical microscope (×400) and then digitized by a charge-coupled device camera on a computer monitor.

To quantify retinal damage induced by transient ischemia, we measured changes in the thickness of the retina by using the method described by Hughes.31 Thickness of the inner plexiform layer and of the overall retina from outer to inner limiting membrane was measured.

**Statistical Analysis**

All values are mean±SEM. The data were analyzed by repeated-measure ANOVA, with post hoc comparisons tested by the Fisher protected least significant difference procedure. Differences were considered statistically significant when the probability values were <0.05.

**Results**

**Time Course of Physiological Variables and Major Retinal Vessel Diameters**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>4h</th>
<th>12h</th>
<th>24h</th>
<th>48h</th>
<th>72h</th>
<th>Argatroban-Treated Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP</td>
<td>119.0±4.7</td>
<td>117.0±9.4</td>
<td>141.7±6.5†</td>
<td>125.7±9.8</td>
<td>172.1±6.9*</td>
<td>141.3±6.4†</td>
<td>125.8±10.8</td>
</tr>
<tr>
<td>HR</td>
<td>286.7±4.8</td>
<td>290.4±9.4</td>
<td>297.8±12.6</td>
<td>294.3±12.4</td>
<td>287.8±10.7</td>
<td>258.7±6.1*</td>
<td>286.2±11.1</td>
</tr>
<tr>
<td>WBC</td>
<td>9.5±1.4</td>
<td>7.5±0.7</td>
<td>8.0±0.7</td>
<td>7.7±1.4</td>
<td>7.4±0.9</td>
<td>6.1±0.4†</td>
<td>5.7±1.4</td>
</tr>
<tr>
<td>PLT</td>
<td>754±22</td>
<td>905±80</td>
<td>856±88</td>
<td>1149±35</td>
<td>949±52</td>
<td>1016±42</td>
<td>851±67</td>
</tr>
<tr>
<td>AD</td>
<td>16.7±0.1</td>
<td>18.4±0.3*</td>
<td>19.2±1.0†</td>
<td>22.3±1.2*</td>
<td>20.4±0.7</td>
<td>18.8±0.7</td>
<td>16.4±0.7§</td>
</tr>
<tr>
<td>VD</td>
<td>31.7±0.4</td>
<td>34.5±1.2</td>
<td>37.1±1.0*</td>
<td>47.5±1.8*</td>
<td>34.2±0.7*</td>
<td>28.3±0.8*</td>
<td>34.5±1.6</td>
</tr>
</tbody>
</table>

BP indicates systolic blood pressure (mm Hg); HR, heart rate (bpm); WBC, peripheral leukocyte count (10⁹/L); PLT, peripheral platelet count (10⁹/L); AD, arterial diameter (μm); VD, venous diameter (μm).

Values are mean±SEM. *P<0.01, †P<0.05 compared with control rats. §P<0.01, §P<0.05 compared with vehicle-treated rats.
retinal vessels. No rolling or adherent platelets were observed along the major retinal vessels in the nonoperated control rats. In the vehicle-treated group, some platelets were observed slowly rolling among many free-flowing platelets along major retinal veins but not along any major retinal arteries (Figure 3A). In the vehicle-treated group, some platelets were observed rolling along the venous walls 4 hours after reperfusion. The number of rolling platelets increased substantially and peaked at 12 hours (36.5±10.0 cells/min). Rolling platelets were inhibited significantly in the argatroban-treated group compared with the vehicle-treated group (P<0.0048; Figure 3B). The maximum number of rolling platelets at 12 hours was significantly reduced to 8.2% in the argatroban-treated group compared with the vehicle-treated group (P=0.0078). In the vehicle-treated group, platelets adherent to the venous walls increased after reperfusion and peaked at 12 hours (12.7±1.2 cells/min). However, platelet adhesion was inhibited significantly in the argatroban-treated group compared with the vehicle-treated group (P=0.0023; Figure 3C). Moreover, the maximum number of adherent platelets at 12 hours was significantly reduced to 21.1% in the argatroban-treated group compared with the vehicle-treated group (P<0.0001).

**P-Selectin and ICAM-I Gene Expression**

The levels of gene expression are shown as a ratio to the average values of nonoperated control rats (Figure 4). ICAM-I mRNA expression was upregulated in the vehicle-treated group and was significantly suppressed in the argatroban-treated group at 6 hours after reperfusion (Figure 2).
Histologic Evaluation

Histologic examination showed a decrease in retinal thickness of operated rats whether they were treated with argatroban or not. The decrease in retinal thickness was more severe in the inner than outer retina. Although retinal thickness was reduced in both groups, it was significantly better preserved in the argatroban-treated group than in the vehicle-treated group (P=0.0002; Figure 5). Furthermore, the protective effect was more substantial in the inner retina. The thickness of the inner plexiform layer in rats treated with argatroban was 183% of that in the vehicle-treated group (P=0.0002).

Discussion

In the current study, histologic findings indicated a protective effect of argatroban against retinal injury after transient retinal ischemia. Our results demonstrated that argatroban could also suppress leukocyte- and platelet–endothelial cell interactions in the postischemic rat retina. The expression of mRNA of P-selectin and ICAM-1 was suppressed significantly in the argatroban-treated retina. On the basis of these findings, we suggest that argatroban might attenuate neural injury after transient ischemia, not only by inhibiting coagulation but also by inhibiting inflammatory reactions mediated by accumulated leukocytes and platelets.

Argatroban is the only direct thrombin inhibitor that is now in use clinically. Other direct thrombin inhibitors, like hirudin and hirulog, are proteins, so their antigenicity makes it
difficult to use them clinically. Heparin is a clinically used drug. However, to express its antithrombotic ability, heparin has to form a complex with antithrombin III. Moreover, because the effect of heparin is irreversible, the dose must be strictly individualized for each patient. In the current study, we used osmotic pumps to administer argatroban continuously for ≈24 hours after ischemia induction because the half-life of argatroban is ≈30 minutes. However, this short half-life allowed us to use this drug safely.

In our study, histologic examination demonstrated the protective effect of argatroban on ischemia-induced retinal damage. Many previous studies have shown that argatroban attenuates neuronal degeneration after forebrain transient ischemia.6,7 In those studies, cerebral blood flow recovered significantly better in the argatroban-treated group than in the vehicle-treated group. Most investigators believe that argatroban exerts its protective effects primarily through inhibition of coagulation after transient cerebral ischemia, resulting in improvement of cerebral blood flow and inhibition of vascular permeability.

Many reports have suggested that leukocytes play a major role in inflammatory injury after transient ischemia.8,9 When endothelial cells are activated by ischemia, adhesion molecules are expressed on the endothelial cells and lead to leukocyte–endothelial cell interaction through a multistep process.12 In this study, argatroban inhibited the mRNA expression of these adhesion molecules after transient retinal ischemia. The suppressed leukocyte–endothelial cell interaction in the postischemic retina of argatroban-treated rats was associated with the suppressed expressions of these adhesion molecules.

Platelets also interact with activated endothelial cells to accumulate in the area of injury.17,18 Thrombin activates endothelial cells and promotes the expression of P-selectin on them. In vivo studies have shown that platelets can roll on activated endothelium through P-selectin expressed on the endothelial cells17,18 and participate not only in coagulation but also in the inflammatory reaction in postischemic tissue. In addition, 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.20–22 Moreover, platelets recruit leukocytes to injured regions through the expression of adhesion molecules on their surfaces or via the production of cytokines.23,24 Suppressed platelet–endothelial cell interactions in the argatroban-treated group would thus contribute in part to the neuroprotective effects of argatroban.

In conclusion, we have demonstrated that argatroban can inhibit platelet–endothelial cell interactions and leukocyte–endothelial cell interactions in retinal tissue that has been injured by transient ischemia. These results suggest that argatroban attenuates ischemia/reperfusion injury not only by inhibiting coagulation but also by inhibiting inflammatory reactions mediated by leukocytes and platelets.

Acknowledgment
This study was supported by a grant-in-aid for scientific research from the Ministry of Education, Science, and Culture, Tokyo, Japan (to J.K. and Y.H.).

References


Argatroban Attenuates Leukocyte- and Platelet-Endothelial Cell Interactions After Transient Retinal Ischemia
Shinsuke Miyahara, Junichi Kiryu, Akitaka Tsujikawa, Hideto Katsuta, Kazuaki Nishijima, Kazuaki Miyamoto, Kenji Yamashiro, Atsushi Nonaka and Yoshihito Honda

Stroke. published online July 17, 2003;
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/early/2003/07/17/01.STR.0000083052.01361.3D.citation

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org/subscriptions/