Effect of Argatroban on Microthrombi Formation and Brain Damage in the Rat Middle Cerebral Artery Thrombosis Model

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ABSTRACT—Ischemic cerebral infarcts induce hypercoagulation and microthrombosis in the surrounding region, thus leading to vascular occlusion. We determined whether microthrombi contribute to the spreading of ischemic lesions following thrombotic middle cerebral artery (MCA) occlusion and also determined whether argatroban, a selective thrombin inhibitor, reduces the formation of the microthrombi and the area of the ischemic lesions. The rat left MCA was occluded by a platelet-rich thrombus formed following the photochemical reaction between rose bengal and green light. Microthrombi were histologically identified in the left hemisphere. The extent of ischemic lesions and microthrombi containing fibrin increased in a time-dependent manner after MCA occlusion. Argatroban inhibited the formation of microthrombi up to 3 hr after MCA occlusion; beyond 3 hr, it was ineffective. Argatroban also significantly (P<0.01) reduced the size of ischemic cerebral lesions at 6 hr after MCA occlusion. It is concluded that the formation of microthrombi contributes to the progression of ischemic lesions in the early stage. It is likely that thrombin generated following thrombotic MCA occlusion contributes to the progression of ischemic lesions by promoting the formation of microthrombi. Argatroban can reduce the formation of microthrombi and ischemic lesions in the early stage.

Keywords: Thrombin, Argatroban, Microthrombosis, Thrombotic middle cerebral artery occlusion

The progression of focal cerebral infarction is dependent on numerous factors (1, 2). Using an autoradiographic technique with iodoantipyrine in an experimental middle cerebral artery (MCA) occlusion model in rats, Nagasawa and Kogure (3) have reported that the ischemic lesions extend from the ischemic core to the surrounding tissues and regional cerebral blood flow decreased in a time-dependent manner. In another study, Garcia et al. (4) have shown progression of ischemic lesions between 30 min and 6 hr after MCA occlusion in rats.

In humans, two factors thought to be involved in the progression of stroke are hypercoagulation and microthrombi formation, which lead to microvascular occlusion and reduction of local blood flow that causes cerebral infarction. Hypercoagulability of blood has been reported in patients during the acute phase of ischemic stroke (5–7). In ischemic brains of humans and those of experimental animals, microthrombi have been observed in the ischemic areas and might contribute to microcirculatory impairment during ischemia (8–10). The presence of fibrin in these thrombi demonstrates that thrombin was produced after MCA occlusion.

Thrombin is well-known to be a key factor in the hemostatic process, converting fibrinogen to fibrin and inducing platelet activation. It is therefore suggested that impaired microvascular perfusion due to thrombin-induced microthrombi in the area surrounding the ischemic core contributes to the progression of ischemic lesions. Argatroban, a synthetic thrombin inhibitor (11, 12), has been shown to prevent platelet-rich arterial thrombosis (13–18).

In view of the above-described observations, we became interested in investigating the change in the microvascular perfusion from the initial ischemic event to the development of an infarction using the rat MCA thrombosis model. We also investigated the effect of argatroban on the microcirculatory disturbance and the ischemic lesions of this model. Our findings suggest that in this model, thrombin generated subsequent to thrombotic occlusion of the MCA promotes microthrombus
formation, thus contributing to the progression of cerebral infarction.

MATERIALS AND METHODS

Animal preparation

Wistar male rats (SLC, Hamamatsu) weighing 240–260 g were used. The body temperature of the animals was maintained at 37.5 °C with a heating-pad (K-module Model K-20; American Pharmaseal Company, Valencia, CA, USA) during the operation. The MCA thrombosis model in the rat has been described previously (19). In brief, under pentobarbital anesthesia and spontaneous respiration, a catheter for the administration of rose bengal was placed in the femoral vein. The scalp and temporalis muscle were folded over, and a subtemporal craniotomy was performed using a dental drill under an operating microscope to open a 3-mm diameter circular bone window.

Photo-irradiation

The 3-mm diameter circular window was irradiated with green light, and the entire irradiated segment including the proximal end of the lenticulostriate branch became thrombotically occluded. Photo-irradiation with green light (wavelength: 540 nm) was achieved by using a xenon lamp (L4887; Hamamatsu Photonics, Hamamatsu) with a heat absorbing filter and a green filter. The irradiation was directed by a 3-mm diameter optic fiber mounted on a micromanipulator. The head of the optic fiber was placed on the window in the skull base at a distance of 2 mm above the vessel, providing an irradiation dose of 0.62 W/cm². Rose bengal (Wako, Osaka; 20 mg/kg) was injected intravenously. Photo-irradiation was continued for a further 10 min. The photochemical reaction between Rose bengal and green light causes endothelial cell injury followed by platelet adhesion and the formation of a platelet-rich thrombus.

Physiological parameters

In separate preliminary experiments, the physiological parameters were measured just before and 15 min after the MCA occlusion. No significant intergroup differences were noted (Table 1). All rats were operated on under identical conditions during this study.

Histological procedures

Just after and 1, 3, 6 and 24 hr after thrombotic MCA occlusion, the rats were perfused transcardially for 1 min with saline followed by 10% formaldehyde solution for histological observations. The brain was immediately removed and fixed in 10% formaldehyde and processed with paraffin. Brain sections were cut into 1-mm-thick consecutive slices from 2.5 mm anterior to bregma. Each section was then stained with phosphotungstic acid-hematoxylin, which can stain fibrin, and with hematoxylin and eosin for a light microscope study. For immunohistochemical analysis, each section was incubated with 100 μl of goat anti-rat fibrinogen antibody (Organon-Teknika N.V., Turnhout, Belgium; 54.5 μg/ml) for 60 min at room temperature in a humidified chamber. Following three phosphate-buffered saline (PBS) washes, sections were incubated with peroxidase-conjugated rabbit anti-goat immunoglobulins (DAKO, Kyoto) for 30 min at room temperature. Following three PBS washes, antibody-bound peroxidase was detected with 10 mg/ml dimethylaminobenzene containing 0.005% hydrogen peroxide. The sections were washed in tap water for 3 min and counterstained with Mayer's hematoxylin for 3 min. The following immunohistochemical controls were routinely performed: 1) deletion of the primary antibody, 2) deletion of the secondary antibody, 3) alteration of the primary antibody to goat immunoglobulin. After hematoxylin counter-staining, the sections were examined by light microscopy for the presence of peroxidase reaction products. The number of microthrombi was counted at 0.5 and 1 mm posterior from the bregma. The ischemic lesions were identified in 4-μm-thick sections stained with hematoxylin and eosin. Areas of the ischemic lesions were examined for histological features such as vacuolation (sponginess) of the neuropil, diffuse pallor of the eosinophilic background, altered shape and stainability of both neuronal cells and astrocytes. The sizes of ischemic lesions at 0.5 and 1 mm posterior from the bregma were measured using a computerized image analysis system, and the ratio of the size of the lesion to the size of the whole cerebral area was calculated.

Table 1. Physiological parameters before and 15 min after thrombotic MCA occlusion in the rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>pH</th>
<th>PCO₂ (mmHg)</th>
<th>PO₂ (mmHg)</th>
<th>Mean blood pressure (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before MCA occlusion</td>
<td>7.40±0.01</td>
<td>41.2±0.9</td>
<td>82.9±2.2</td>
<td>114±3</td>
</tr>
<tr>
<td>After MCA occlusion</td>
<td>7.42±0.02</td>
<td>40.1±0.8</td>
<td>88.1±1.8</td>
<td>118±3</td>
</tr>
</tbody>
</table>

Reported values are means ± S.E.M. derived from 10 observations.
Fig. 1. Typical photograph of microthrombi of 10 animals on the occluded hemisphere 3 hr after MCA occlusion. The bar indicates 50 μm. a Fibrin-rich microthrombi PTAH stain b Fibrinogen and fibrin detected by anti-rat fibrinogen antibody.
Administration of argatroban

For the group of animals whose brains were removed within 6 hr after thrombotic MCA occlusion, argatroban (40 mg/kg) was administered twice subcutaneously just after and 3 hr after thrombotic MCA occlusion to maintain anticoagulant activity in the plasma up to 6 hr. One group of animals was injected with saline subcutaneously in the same way as the argatroban administration group to use as controls. For the group of animals whose brains were removed 24 hr after thrombotic MCA occlusion, an osmotic pump (Alzet, Palo Alto, CA, USA; 2001D, 8 μl/hr) filled with 40 mg/ml argatroban or saline was implanted in the abdomen just after thrombotic MCA occlusion. The activated partial thromboplastin time was determined using pathrontin (Behringwerke, Tokyo) in all animals at the end of each experiment as an index of plasma coagulability.

Statistical analyses

Data are expressed as means ± S.E.M. Statistical analysis of the number of thrombi was performed with an unpaired Mann-Whitney test for comparison between groups. Statistical analysis of the size of the ischemic lesion was performed with the unpaired Student’s t-test for comparison groups. A P value <0.05 was considered significant.

RESULTS

With phosphotungstic acid hematoxylin staining, fibrin was strongly blue-positive (Fig. 1a). This phosphotungstic acid hematoxylin-positive thrombi was immunoreactive with anti-rat fibrinogen antibody which can react with rat fibrinogen and fibrin (Fig. 1b). The thrombi were only found in the left hemisphere, in the side supplied by the occluded MCA (Fig. 2). Just after thrombotic MCA occlusion, microthrombi appeared on the surface of the brain and then extended to the cortex, the caudoputamen (Fig. 2). In the group without MCA occlusion and photoradiation, the animals were checked for MCA, but no microthrombi were detected (data not shown). The number of fibrin-rich microthrombi in each animal continued to increase until 3 hr after thrombotic MCA occlusion (Fig. 3). At 3 hr, the number of fibrin-rich microthrombi in animals treated with argatroban was significantly (P<0.01) reduced as compared with untreated animals (Fig. 3).

Ischemic lesions were detected using histological techniques. Three hours after thrombotic MCA occlusion, the lesions were confined to the preoptic area and extended to the lateral caudoputamen within 6 hr after thrombotic MCA occlusion. Finally, the ischemic lesions extended into the cortex and caudoputamen 24 hr after thrombotic MCA occlusion (not shown). At 6 hr after thrombotic MCA occlusion, the ischemic lesion in the group treated with argatroban was significantly (P<0.05) smaller as compared with untreated animals (Fig. 4).

In the group treated with argatroban, the activated partial thromboplastin times (APTT) were 66.5±2.7 sec.
at 3 hr, 80.0 ± 3.2 sec at 6 hr, 49.1 ± 2.9 sec at 24 hr and 26.5 ± 0.6 sec, 25.0 ± 0.7 sec, 24.4 ± 0.6 sec at the respective times in each control group (n=6) (P<0.01). Based on APTT prolongation, the effect of argatroban continued up to the end of each experiment.

**DISCUSSION**

In this study, the thrombotic occlusion of MCA was induced by the photochemical reaction between rose Bengal and green light which causes endothelial injury followed by platelet adhesion and formation of a platelet- and fibrin-rich thrombus. The thrombotic occlusion of MCA resulted in ischemic damage to the brain tissues. The extent of ischemic lesions and number of microthrombi containing fibrin increased time-dependently after MCA occlusion. Ischemic lesions increased to the same degree as in other models in which the middle cerebral arteries are occluded by electrocoagulation (4). However, the extent of microthrombi formation in our model was quite different from those in the other models (8–10). Heye et al. have reported that the maximum microthrombus formation was found 7 days after MCA occlusion, and the microthrombi were found in both the occluded and the contralateral hemispheres. In our model, the microthrombi beyond 6 hr after thrombotic MCA occlusion were not detectable because leukocytes infiltrated and therefore the structure of the microvessels was broken.

These microthrombi may contribute to the secondary brain damage caused by MCA occlusion. The microthrombi may be formed from circulating platelets that are activated or aggregated at the damaged middle cerebral artery vessels. It has also been reported that tissue factor, which is the major initiator of the coagulation cascade leading to the generation of thrombin, is present in cerebral tissue (20, 21) and contributes to the no-reflow phenomenon in a baboon model of MCA occlusion (22). Furthermore, it has been reported that interleukin-1β is overexpressed in the brain in response to ischemia (23). Interleukin-1β is a cytokine with procoagulant actions (24). This induced cytokine may form a microthrombus.

The selective thrombin inhibitor argatroban significantly inhibited the formation of microthrombi at 3 hr after thrombotic MCA occlusion, but it was ineffective beyond 3 hr. The inhibitory effect of argatroban on the formation of microthrombi was reflected in a significant reduction in the size of cerebral ischemic lesions 6 hr after thrombotic MCA occlusion, but argatroban did not reduce the extent of lesions observed 24 hr after thrombotic MCA occlusion. The effect of argatroban on microthrombi formation at 6 hr after thrombotic MCA occlusion was observed to be different from its effect on the ischemic lesions at that time; this may be due to the difficulty of detecting the microthrombi formation 6 hr after thrombotic MCA occlusion. Thus, the inhibitory effect of argatroban on the formation of microthrombi suggests that thrombin contributes to ischemic brain damage following thrombotic MCA occlusion.

In this study, we observed that fibrin-rich microthrombi formed within 3 hr following MCA occlusion. It is concluded that in our rat model, following endothelial injury and the thrombotic occlusion of MCA, brain tissue directly supplied by the MCA becomes infarcted. Subsequently, thrombin is generated; it induces the formation of the microthrombi, thus causing further ischemic brain damage by blocking collateral microvessels. Yet, it has been reported that polymorphonuclear leukocytes contribute to post-ischemic perfusion abnormalities (25, 26) and brain edema (27). Further ischemic damage may be caused by other factors such as leukocytes and free radicals (28) that may not be related to thrombin because they were unaffected by argatroban.

**Acknowledgments**

The authors thank Dr. A. Saniabadi, Senior Scientist at Terumo Corp., Kanagawa, Japan for editing the manuscript.

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