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

Hiroshi Kawai#, Kazuo Umemura and Mitsuyoshi Nakashima

Department of Pharmacology, Hamamatsu University School of Medicine, 3600 Handa-cho, Hamamatsu 431–31, Japan

Received May 19, 1995 Accepted July 26, 1995

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 microthrombosis, 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 thrombologically 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.

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

| Parameter                  | pH         | $P_{O_2}$ (mmHg) | $P_{O_2}$ (mmHg) | Mean blood pressure (mmHg) |
|----------------------------|------------|-----------------|-----------------|--------------------------|
| Before MCA occlusion      | 7.40±0.01  | 41.2±0.9        | 82.9±2.2        | 114±3                    |
| After MCA occlusion       | 7.42±0.02  | 40.1±0.8        | 88.1±1.8        | 118±3                    |

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.

Fig. 2. The formation of the microthrombi just after (1), 1 hr after (2), 3 hr after (3), 6 hr after (4) MCA occlusion at 0.5 mm posterior from the bregma. Total of the microthrombi counted in all of 10 rats of each experimental group. Dots show the actual localization of a microthrombus in the brain.

Fig. 3. Effect of argatroban on number of microthrombi. The mean number of microthrombi counted at 0.5 mm and 1 mm posterior from the bregma. Reported values are means ± S.E.M. derived from 10 observed rats after thrombotic MCA occlusion. **P<0.01 vs animals treated with vehicle. ○: Vehicle, ●: Argatroban.
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.

REFERENCES

1 Scheinberg P: The biologic basis for the treatment of acute stroke. Neurology 41, 1867–1873 (1991)
2 Raichle ME: The pathophysiology of brain ischemia. Ann
15 Fitzgerald DJ and Fitzgerald GA: Role of thrombin and thromboxane \( \text{A}_2 \) in reocclusion following coronary thromboly-
sis with tissue-type plasminogen activator. Proc Natl Acad Sci
USA 86, 7585–7589 (1989)

16 Edit JF, Allison P, Noble S, Ashton J, Golino P, McNatt J,
Buja LM and Willerson JT: Thrombin is an important mediator
of platelet aggregation in stenosed canine coronary arteries with
endothelial injury. J Clin Invest 84, 18–27 (1989)

17 Jang IK, Gold HK, Zisking AA, Leinbach RC, Fallon JT and
Collen D: Prevention of platelet-rich arterial thrombosis by
selective thrombin inhibition. Circulation 81, 219–225 (1990)

18 Yasuda T, Gold HK, Yaoita H, Leinbach RC, Guerrero JL,
Jang IK, Holt R, Fallon JT and Collen D: Comparative effects
of aspirin, a synthetic thrombin inhibitor and a monoclonal
antiplatelet glycoprotein IIb/IIIa antibody on ricombinant tis-
sue-type plasminogen activator in a canine preparation. J Am
Coll Cardiol 16, 714–722 (1990)

19 Umemura K, Wada K, Uematsu T and Nakashima M: Evalu-
ation of the combination of a tissue-type plasminogen activator,
SUN9216, and a thromboxane \( \text{A}_2 \) receptor antagonist,
Vapiprost, in a rat middle cerebral artery thrombosis model.
Stroke 24, 1077–1081 (1993)

20 del Zoppo GJ, Yu JQ, Copeland BR, Thomas WS, Schneider-
man J and Morrissey JH: Tissue factor localization in non-
human primate cerebral tissue. Thromb Haemost 68, 642–647
(1992)

21 Fleck RA, Rao LVM, Rapaport SI and Varki N: Localization
of human tissue factor antigen by immunostaining with
monospecific polyclonal anti-human tissue factor antibody.
Thromb Res 57, 765–782 (1990)

22 Thomas WS, Mori E, Copeland BR, Yu JQ, Morrissey JH and
del Zoppo GJ: Tissue factor contributes to microvascular
defects after focal cerebral ischemia. Stroke 24, 847–854 (1993)

23 Liu T, McDonnell PC, Young PR, White RF, Siren AL,
Hallenbeck JM, Barone FC and Feuerstein GZ: Interleukin-1β
mRNA expression in ischemic rat cortex. Stroke 24, 1746–1751
(1993)

24 Dinarello CA: Biology of interleukin-1. FASEB J 2, 108–115
(1988)

25 del Zoppo GJ, Schmid-Schonbein GW, Mori E, Copeland BR
and Chang CM: Polymorphonuclear leukocytes occlude capil-
laries following middle cerebral artery occlusion and reperfu-
sion in baboons. Stroke 22, 1276–1283 (1991)

26 Mori E, del Zoppo GJ, Chambers JD, Copeland BR and Arfors
KE: Inhibition of polymorphonuclear leukocyte adherence
suppresses no-reflow after focal cerebral ischemia in baboons.
Stroke 23, 712–718 (1992)

27 Shiga Y, Onodera H, Kogure K, Yamasaki Y, Yashima Y,
Syozuhara H and Sendo F: Neutrophil as a mediator of
ischemic edema formation in the brain. Neurosci Lett 125,
110–112 (1991)

28 Pulcinelli W: The ischemic penumbra in stroke. Sci Am 2,
16–25 (1995)