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Evaluation of a GPIIb/IIIa antagonist YM337 in a primate model of middle cerebral artery thrombosis

Seiji Kaku [a,*], Kazuo Umemura [a], Atsuhiro Mizuno [a], Shinya Yano [b], Ken-ichi Suzuki [b], Tomihisa Kawasaki [b], Mitsuyoshi Nakashima [a]

[a] *Department of Pharmacology, Hamamatsu University School of Medicine, Hamamatsu 431-31, Japan*
[b] *Institute for Drug Discovery Research, Yamanouchi Pharmaceutical, Tsukuba City, Ibaraki 305, Japan*

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Abstract

We compared the antithrombotic effect of anti-GPIIb/IIIa antibody Fab fragment YM337 with that of a thromboxane A_2 synthetase inhibitor, sodium ozagrel. With the monkeys under halothane anesthesia, the right middle cerebral artery was observed via a transorbital approach without cutting the dura mater. Photoillumination (wavelength 540 nm) was applied to the middle cerebral artery, and then rose bengal (20 mg kg^{-1}) was administered intravenously. The experimental drugs were intravenously injected 15 min before rose bengal injection and followed by continuous infusion for 3 h after dye injection. The thrombotic occlusion induced by this photochemical reaction in monkey middle cerebral artery was reproducible. YM337 significantly prolonged the time to first occlusion and the total time of arterial patency during the 3-h observation period after dye injection. In contrast, sodium ozagrel had no significant effect. YM337 but not sodium ozagrel significantly inhibited ex vivo ADP-induced platelet aggregation. However, while sodium ozagrel significantly inhibited the thromboxane B_2 generation accompanying arachidonic acid-induced platelet aggregation, YM337 had no effect on this variable. Neurological deficit in the YM337-treated animals was significantly milder than that in the control group. The area of infarct in the YM337 treatment animals was smaller than that in the control group. The novel selective GPIIb/IIIa antagonist YM337 was effective in ameliorating the decrease in patency of the middle cerebral artery and reducing the area of cerebral infarction in monkeys. © 1998 Elsevier Science B.V.

Keywords: Antibody, humanized; Platelet GPIIb/IIIa; Thrombosis, cerebral; Monkey

1. Introduction

Many experimental models of ischemic stroke have been developed in a wide variety of animal species. We previously developed a model of cerebral ischemia based on middle cerebral artery thrombosis induced by photochemical reaction in rats (Umemura et al., 1993). Although nonhuman primates are considered extremely valuable in the study of ischemic stroke because the behavior, motor and sensory integration, amount of neocortex, and construction of the cerebral vasculature are similar to that in man (Garcia, 1984), an ideal monkey model for the investigation of thrombotic stroke has not been established. In this study, we applied the photochemically induced thrombosis model to squirrel monkeys to produce a more suitable model for the investigation of human thrombotic stroke.

Platelet aggregation, which plays an essential role in arterial thrombosis, is widely implicated in the pathogenesis of thrombotic stroke. Treatment with antiplatelet agents has reduced morbidity and mortality in patients with ischemic stroke (Vicari et al., 1987; Born, 1990; Rothrock and Hart, 1991; Diener et al., 1996). Hyperaggregability of platelets has been reported in patients with transient ischemic attacks and cerebral thrombosis (Couch and Hassanein, 1976; Andersen and Gormsen, 1977; Dougherty et al., 1977; Uchiyama et al., 1994). However, the efficacy of antiplatelet agents for acute ischemic stroke has not been established.

Currently available antiplatelet agents such as aspirin show some efficacy in the treatment of arterial thrombotic

* Corresponding author. Institute for Drug Discovery Research, Yamanouchi Pharmaceutical, 21 Miyukigaoka, Tsukuba City, Ibaraki 305, Japan. Tel.: +81-298-54-1572; fax: +81-298-52-2955; e-mail: kaku.seiji@yamanouchi.co.jp

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disorders, but results have generally fallen short of expectations. While aspirin prevents platelet thromboxane A_2 generation and decreases ex vivo platelet aggregation, platelet aggregation still occurs in response to many agonists, such as ADP, thrombin and platelet-activating factor. The final common step in the formation of a platelet aggregate is the linking of adjacent platelets by fibrinogen binding to the platelet glycoprotein IIb/IIIa (GPIIb/IIIa) (Plow and Ginsberg, 1989; Phillips et al., 1988). This step is thought to represent an excellent target for the development of antiplatelet agents. In animal experiments, GPIIb/IIIa antagonist prevents platelet thrombus formation more effectively than aspirin (Coller et al., 1991; Coller, 1992). Further, the GPIIb/IIIa antagonist abciximab (the Fab fragment of human/murine chimeric monoclonal antibody 7E3, ReoPro™) has been approved by the US Food and Drug Administration as well as by several European and Scandinavian countries for use in patients undergoing high-risk coronary artery angioplasty and atherectomy. Recently, ReoPro has been used to treat basilar artery thrombosis and has prevented rethrombosis (Wallace et al., 1997). This result suggests the usefulness of GPIIb/IIIa antagonists in treating human stroke.

Recently, we developed the murine anti-GPIIb/IIIa monoclonal antibody C4G1 (Yano et al., 1994), which is highly specific to GPIIb/IIIa complex, and succeeded in humanizing it by complementarity determining region (CDR) grafting (Co et al., 1994). YM337, the Fab fragment of humanized C4G1, showed a potent antiplatelet effect in rhesus monkeys ex vivo (Kaku et al., 1996). YM337 also prevented platelet thrombus formation in the microvessels of squirrel monkey mesentery (Kaku et al., 1996). Because YM337 reacts with human platelets and platelets from rhesus and squirrel monkeys but not with those from mice, rats, guinea pigs or dogs, the squirrel monkey was selected as the experimental animal in this study.

In the present study, we investigated the effect of YM337 in comparison with that of sodium ozagrel, a selective thromboxane A_2 synthetase inhibitor (Naito et al., 1983) in clinical use for the acute stage of cerebral thrombosis in Japan, on photochemically induced thrombosis in the middle cerebral artery of squirrel monkeys.

2. Materials and methods

2.1. Animal preparation

Squirrel monkeys (*Saimiri sciureus*) weighing 535 to 915 g were lightly anesthetized by intramuscular injection of 20 mg kg^{-1} ketamine hydrochloride. Endotracheal intubation was done and anesthesia maintained with halothane at a concentration varying between 0.5% and 1.5%. The animals were placed in the supine position on a heating pad (American Pharmseal, K-module Model K-20). Catheters were inserted in the femoral artery and vein for the measurement of blood gases, continuous recording of blood pressure and heart rate, and for the administration of drug, and a rectal thermometer was inserted for constant monitoring of body temperature. The transorbital approach to the right middle cerebral artery was performed according to the method of Hudgins et al. (Hudgins and Garcia, 1970). The orbital contents were dissected and excised. With the help of a Zeiss operating microscope, orbital craniotomy was performed using a dental drill to open a oval bony window. This permitted visualization of the intracranial dura and the right middle cerebral artery could be observed without cutting the dura mater. Thrombus was induced by photochemical reaction according to the method of Umemura et al. (1993). Briefly, the window was irradiated with green light (wave length 540 nm) achieved by use of a xenon lamp (L4887, Hamamatsu Photonics, Hamamatsu, Japan) 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 probe of a pulsed doppler flowmeter (Model PDV-20, Crystal Biotech America, USA) was placed on the middle cerebral artery to measure middle cerebral artery blood flow. When a steady baseline flow was obtained, irradiation was started, and rose bengal (20 mg kg^{-1}) was injected intravenously. Photoirradiation was continued for a further 20 min. Blood flow in the middle cerebral artery was continuously monitored for 3 h after rose bengal injection. The middle cerebral artery was considered to be occluded when the flow monitor indicated that blood flow had completely stopped. The time from injection of rose bengal to the cessation of blood flow was recorded as the middle cerebral artery occlusion time. The dura was then covered with a moist gelatin sponge, the wound was closed, and the eyelids were sutured together. All animal treatments were approved by the Ethics Committee for Laboratory Animal Experiments at the Hamamatsu University School of Medicine.

2.2. Experimental procedure

The experimental protocol is summarized in Fig. 1. Drug administration was started 15 min before rose bengal injection and continued for 3 h after injection of the dye. Five treatment groups of five monkeys each were studied: a saline group with intravenous bolus injection of 1 ml kg^{-1} saline followed by 5 ml kg^{-1} h^{-1} infusion; a low-dose YM337 group with intravenous bolus injection of 0.5 mg kg^{-1} YM337 followed by 3 μg kg^{-1} min^{-1} infusion; a high-dose YM337 group with intravenous bolus injection of 1 mg kg^{-1} YM337 followed by 6 μg kg^{-1} min^{-1} infusion; a low-dose sodium ozagrel group with intravenous bolus injection of 5 mg kg^{-1} sodium ozagrel followed by 50 μg kg^{-1} min^{-1} infusion; and a high-dose sodium ozagrel group with intravenous bolus injection of

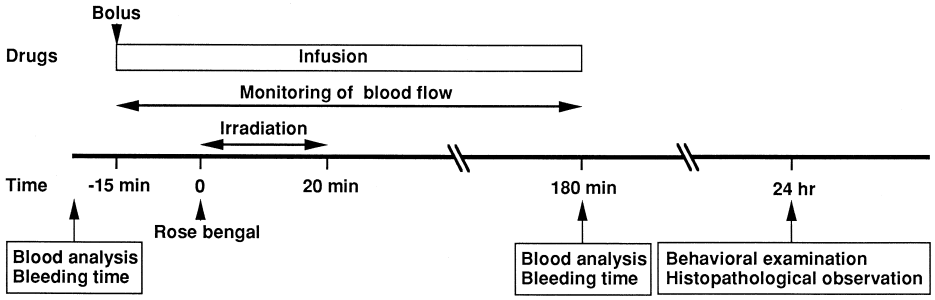

Fig. 1. Schematic representation of the study protocol.

10 mg kg^{-1} sodium ozagrel followed by 100 μg kg^{-1} min^{-1} infusion.

2.3. Neurological examination

About 24 h after surgery, monkeys were examined for evidence of neurological deficit. Deficits were graded using a simple five-point scale according to the method of Crowell et al. (1970).

Grade 0 = no deficit: normal climbing.
Grade 1 = mild deficit: abnormal climbing, decreased dexterity of the contralateral hand.
Grade 2 = moderate deficit: cannot climb, abnormal walking.
Grade 3 = severe deficit: cannot walk, abnormal standing.
Grade 4 = cannot stand.
Grade 5 = death.

2.4. Histopathological observation

About 24 h after surgery, monkeys were anesthetized with sodium pentobarbital (20 mg kg^{-1}) and transcardiac perfusion was carried out with buffered formalin. Brains were removed and additionally fixed by immersion in buffered formalin. Each brain was coronally sectioned into 5-mm thick slices from the frontal lobe. Seven consecutive slices (10 to 40 mm from the frontal lobe) were then embedded in paraffin, and sections were cut and then stained with hematoxylin and eosin stain and Nissl's method. For each animal, the area of infarction was measured using a computerized image analysis system (Model VM-30, Olympus, Tokyo, Japan), and the ratio of the infarction area to the whole area of the corresponding cerebrum was calculated.

2.5. Platelet aggregation assay

Platelet-rich plasma and platelet-poor plasma was prepared by centrifugation of citrate-anticoagulated blood. Platelet counts in platelet-rich plasma were determined with an automatic cell counter (MEK-6158, Nihon Koden, Tokyo, Japan), and adjusted to a count of 3×10^5 platelets μl^{-1} with platelet-poor plasma. Platelet aggregation in platelet-rich plasma was measured using an aggregometer (NBS Hematracer model 601, Niko Bioscience, Tokyo, Japan), by recording the increase in light transmission through a stirred suspension of platelet-rich plasma maintained at 37°C for 5 min. Aggregation was induced by ADP at 20 μM or arachidonic acid at 0.5 mM.

2.6. Bleeding time

Bleeding time of the monkeys was measured using a standard clinical procedure. A pneumatic cuff was inflated to 40 mm Hg on the bicep of the monkey. Bleeding time was measured in the forearm with a spring-loaded blade system (Simplate R, Organon Teknika, Durham, USA), avoiding major subcutaneous veins. Blood coming from the incision was blotted with filter paper every 30 s until blood no longer stained the filter paper.

2.7. Thromboxane B_2 assay

Released thromboxane A_2 was extracted from plasma samples as described elsewhere (Yamaki and Oh-ishi, 1989). Extraction yield was more than 80% in all experiments. The concentration of extracted thromboxane A_2 was measured as the concentration of thromboxane B_2 by ELISA (Cayman Chemical, Ann Arbor, MI). Measurement was done according to the manufacturer's instructions.

2.8. Plasma concentration of unbound YM337

Concentrations of unbound YM337 in plasma were measured by ELISA as described previously (Kaku et al., 1996).

2.9. Occupancy of GPIIb / IIIa

Occupancy of GPIIb/IIIa was measured by ELISA. Briefly, 96-well microtiter plates were coated with anti-GPIIIa antibody (B6A3) (Yano et al., 1994) at 5 μg ml^{-1}. The plates were blocked with 35 mg ml^{-1} bovine serum albumin. Diluted samples in which platelet-rich plasma was solubilized with 1/10 volume of 10% Triton X-100

were poured into the wells and incubated at room temperature for 2 h. After washing several times, the plates were incubated with 4000-fold diluted peroxidase-conjugated goat anti-human $F(ab')_2$ (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at room temperature for 1 h. After washing several times, the amount of bound enzyme was finally quantified using 2,27-azido-bis(3-ethylbenzthazoline-6-sulfonic acid) (ABTS; Bio-Rad, Richmond, CA) and color absorbance was measured at 414 nm. Background values were measured by predose samples (0% occupancy). Complete blockade (100% of occupancy) was measured using predose samples to which saturated amounts of YM337 were added. Occupancy of the samples was calculated from these values.

2.10. Statistical analysis

Data are expressed as mean ± S.E.M. The groups were compared using one-way analysis of variance (ANOVA) while significant differences were determined using Dunnett multiple range test. In the bleeding time and neurological deficit study, the groups were compared using the Kruskal–Wallis *H*-test, with the Steel test used for significant differences. A P value less than 0.05 was considered significant.

3. Results

Table 1 summarizes the preischemic physiological parameters obtained before treatment in each group. There were no significant intergroup differences in body weight, mean arterial pressure, blood gases, pH, blood cell counts and body temperature in this experiment. However, heart rate was significantly higher in the high dose YM337 group.

3.1. Patency of middle cerebral artery

The time course of patency of the middle cerebral artery in individual animals is schematically represented in Fig. 2. The time to first occlusion after rose bengal injection (time to occlusion) and the total time of arterial patency during the 3-h observation period (total patency time) are shown in Table 2. YM337 prolonged the time to occlusion in a dose-dependent manner and was statistically significant in the high-dose group compared with the saline group ($P < 0.01$). Sodium ozagrel did not prolong the time to occlusion. YM337 also prolonged the total patency time in a dose-dependent manner and this was statistically significant in the high-dose group compared with the saline group ($P < 0.05$). Sodium ozagrel prolonged the total patency time about two-fold over that of the saline group but the difference was not statistically significant. At about 24 h after injection of rose bengal, the treated middle cerebral artery were no longer occluded in any animal.

3.2. Platelet aggregation, thromboxane generation and bleeding time

The results for ex vivo platelet aggregation, thromboxane generation and bleeding time are shown in Table 3. ADP-induced platelet aggregation was significantly inhibited with a high dose of YM337 at 3 h after rose bengal injection ($P < 0.001$) but remained normal in the sodium ozagrel groups. Platelet aggregation induced with arachidonic acid was decreased to less than 30% of baseline mean value in all treatment groups. This decrease was statistically significant in the high-dose sodium ozagrel group compared with the saline group ($P < 0.05$). Sodium ozagrel also inhibited the thromboxane B_2 generation accompanying arachidonic acid-induced platelet aggregation. YM337 had no effect on this variable. Template bleeding

Table 1
Pretreatment physiological variables

Parameters	Saline group	YM337 group		Sodium ozagrel group	
		Low dose	High dose	Low dose	High dose
Body weight (g)	712 ± 68	700 ± 30	694 ± 68	736 ± 52	694 ± 45
pO_2 (mmHg)	115 ± 14	132 ± 2	129 ± 8	111 ± 5	105 ± 11
pCO_2 (mmHg)	36.1 ± 0.7	38.2 ± 0.9	36.6 ± 2.0	37.7 ± 1.0	37.6 ± 1.8
pH	7.37 ± 0.01	7.36 ± 0.01	7.38 ± 0.01	7.38 ± 0.01	7.38 ± 0.02
Rectal temperature (°C)	38.5 ± 0.2	38.3 ± 0.3	38.5 ± 0.3	38.7 ± 0.1	38.5 ± 0.1
RBC ($\times 10^4$ μl^{-1})	732 ± 35	770 ± 14	757 ± 19	747 ± 29	739 ± 17
PLT ($\times 10^4$ μl^{-1})	47.9 ± 5.3	56.1 ± 4.1	49.5 ± 3.5	50.9 ± 6.3	45.6 ± 4.0
HCT (%)	50.9 ± 2.6	52.6 ± 0.71	53.1 ± 1.3	51.7 ± 2.3	52.0 ± 1.0
MBP (mmHg)	72 ± 5	80 ± 6	78 ± 5	84 ± 6	77 ± 4
HR (beats min^{-1})	237 ± 12	267 ± 11	280 ± 7[a]	274 ± 11	257 ± 13

[a] $P < 0.05$ compared with saline group.
RBC indicates red blood cell; PLT, platelet; HCT, hematocrit; MBP, mean blood pressure; and HR, heart rate. Values are mean ± S.E.M. ($n = 5$).

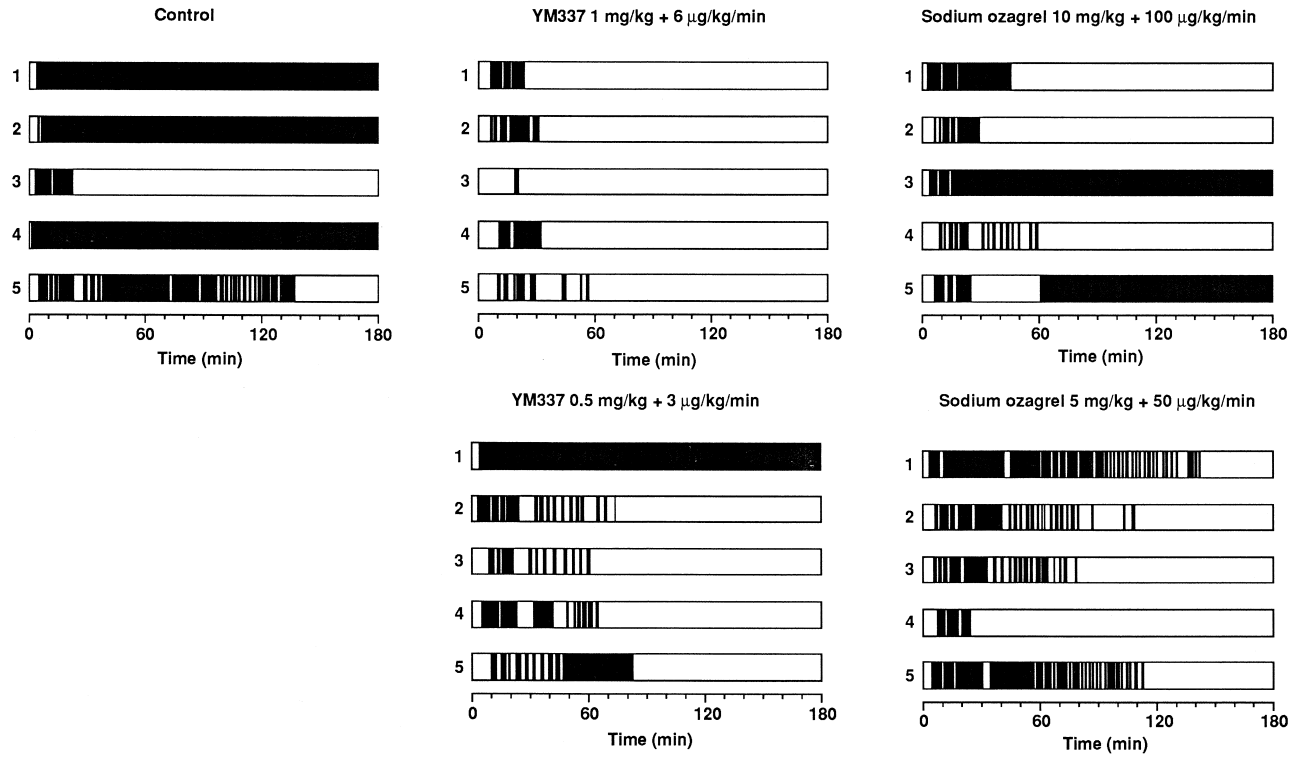

Fig. 2. Schematic representation of the patency status of the middle cerebral artery in the individual animals after photochemical reaction. Open portion of each bar indicates a patent vessel; closed portion of each bar indicates a closed vessel.

times at baseline were approximately 3 min. Bleeding time was prolonged more than three times the pretreatment value in two animals in the high-dose sodium ozagrel group at 3 h after rose bengal injection. However, prolongation of bleeding time was not statistically significant in any treated group compared with the saline group.

Table 2
Posttreatment time to occlusion and total patency time

Dose groups		Occlusion time (min)		Total patency time (min)	
		Mean ± S.E.M.	Range	Mean ± S.E.M.	Range
Saline		3.8 ± 0.63	1.5–5.3	54.4 ± 32.1	2.5–162
YM337	Low dose	6.3 ± 1.4	3.3–10.1	119 ± 29.3	4–162
YM337	High dose	10.5 ± 2.3[a]	6.2–18.7	167 ± 3.05[b]	160–178
Sodium ozagrel	Low dose	5.6 ± 0.73	3.5–7.7	131 ± 12.1	98–167
Sodium ozagrel	High dose	5.7 ± 1	3–8.4	103 ± 32.3	6.2–162

[a] $P < 0.01$ compared with saline group.
[b] $P < 0.05$.
Values are mean ± S.E.M. ($n = 5$).

Table 3
Posttreatment platelet aggregation, bleeding time and thromboxane B_2 generation

Dose groups		Platelet aggregation (% of pretreatment)		Bleeding time (-fold of pretreatment)	TXB_2 generation (% of pretreatment)
		ADP	Arachidonic acid		
Saline		88 ± 4.2	69.6 ± 21.6	1 ± 0.04	82 ± 6.9
YM337	Low dose	70.5 ± 3.7	26.4 ± 17.1	1.3 ± 0.17	78.3 ± 11
YM337	High dose	41.0 ± 8.2[a]	18.1 ± 16.4	1.3 ± 0.25	79.5 ± 6.0
Sodium ozagrel	Low dose	96.9 ± 1.6	19.9 ± 14.2	1.7 ± 0.37	4.1 ± 0.92[a]
Sodium ozagrel	High dose	94.6 ± 9.3	6.9 ± 4[b]	2.8 ± 0.86	5.1 ± 1.5[a]

[a] $P < 0.001$ compared with saline group.
[b] $P < 0.05$.
Values are mean ± S.E.M. ($n = 5$).

Table 4
Posttreatment behavioral deficit

Dose groups		Grade of behavioral deficit					P[a]
		0	1	2	3	4	
Saline		0	1	3	0	1	
YM337	Low dose	1	2	1	0	1	NS
YM337	High dose	4	1	0	0	0	< 0.05
Sodium ozagrel	Low dose	2	1	2	0	0	NS
Sodium ozagrel	High dose	1	4	0	0	0	NS

[a] P compared with saline group.
NS: not significant.

3.3. Plasma concentration of YM337 and occupancy of GPIIb / IIIa

The plasma concentrations of unbound YM337 were 3.54 ± 0.96 μg ml^{-1} (high dose) and 0.74 ± 0.25 μg ml^{-1} (low dose) at 3 h after photochemical reaction. YM337 treatment resulted in a reduction of the number of free GPIIb/IIIa receptors and $59.2 \pm 5.7\%$ (high dose) and $35.7 \pm 2.6\%$ (low dose) saturation of the GPIIb/IIIa receptors at 3 h after photochemical reaction.

3.4. Cerebral infarction and neurological examination

The results of neurological examination at about 24 h after rose bengal injection are shown in Table 4. There were no deaths under these experimental conditions within 24 h posttreatment. All animals were much less combative than normal. The neurological deficits of the high-dose YM337-treated animals were significantly milder than those in the control group ($P < 0.05$). Sodium ozagrel also decreased the neurological deficits but not significantly. The areas infarction in seven slices are shown in Fig. 3. Infarcts involved the head of the caudate nucleus, the anterior limb and genu of the internal capsule, putamen and external capsule. In some cases, the area around the sylvian fissure was infarcted. Sodium ozagrel had no effect on infarct size and YM337 had a minimal but significant effect in the slice 2 region of the hemisphere, which involved the anterior limb of the internal capsule and the head of the caudate nucleus ($P < 0.05$ compared with saline group).

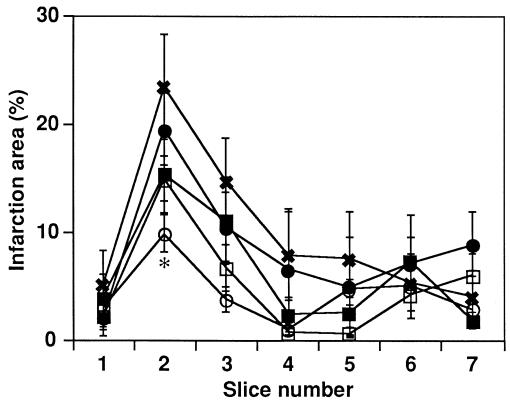

Fig. 3. The areas of ischemic damage in seven coronal slices in saline (control group) (x), YM337 (●; low dose, ○; high dose) and sodium ozagrel (▲; low dose, △; high dose) treated animals. Values are the mean ± S.E.M. for each group of five animals. * $P < 0.05$ vs. saline group.

4. Discussion

Various experimental models of ischemic stroke caused by middle cerebral artery occlusion have been developed using clipping, ligation or inflation of a cuff in monkeys (Hudgins and Garcia, 1970; Crowell et al., 1970; Sundt et al., 1969; Symon et al., 1975; Jones et al., 1981; Del Zoppo et al., 1986). However, these types of induced middle cerebral artery occlusion cannot be used for investigation of the effect of antithrombotic or thrombolytic drugs. In the present study, we aimed to produce a monkey model of middle cerebral artery occlusion resulting from thrombosis induced by photochemical reaction. The induction method used in this study, which has been reported previously (Nakayama et al., 1988), causes the formation of active oxygen species that damage the endothelium. Consequently, platelets adhere to and aggregate on the damaged vessel, resulting in occlusion. An advantage of this method is that middle cerebral artery occlusion can be achieved without cutting the dura mater. We used this method to produce reproducible thrombotic occlusion in monkey middle cerebral artery. Interestingly, cyclical reperfusion and reocclusion were observed in some animals; the importance of these phenomena in ischemic cerebral damage, however, is at present unknown.

After photoirradiation, we evaluated the effect of YM337, the Fab fragment of the humanized monoclonal antibody C4G1 which specifically reacts with GPIIb/IIIa complex. YM337 blocked fibrinogen binding to GPIIb/IIIa on platelet and inhibited platelet aggregation, thus prolonging the time to occlusive thrombus formation in the monkey middle cerebral artery. YM337 also improved the patency status of the middle cerebral artery during the 3-h observation period after photochemical reaction. These results suggest that blockade of GPIIb/IIIa is a beneficial approach to preventing cerebral artery thrombosis.

YM337 also decreased the neurological deficit and reduced the area of ischemic cerebral damage. However, these effects resulted when YM337 was administered before middle cerebral artery injury. To confirm the clinical therapeutic potential of YM337, an experiment in which YM337 is administered after thrombus formation may be needed.

No significant prolongation of bleeding time was seen with any drug used in this study. Even at a dose which significantly inhibited ex vivo platelet aggregation, prolonged the time to occlusive thrombus formation, and

improved patency status, YM337 at the doses studied was not associated with a prolongation in template bleeding time.

Aspirin is widely used as standard preventive therapy in patients with acute myocardial infarction, transient ischemic attacks or stroke (Hennekens, 1990; Koller, 1991). Aspirin is an irreversible, noncompetitive inhibitor of cyclooxygenase and acts as an antiplatelet agent through the prevention of thromboxane A_2 generation in platelets. However, aspirin also prevents the generation of prostaglandin I_2 in endothelial cells. To exclude this possibility, the selective thromboxane A_2 synthetase inhibitor sodium ozagrel was used in this experiment. The antithrombotic effect of sodium ozagrel in arachidonate-induced thrombosis models has been reported by Hiraku et al. (1986). In this study, sodium ozagrel prevented the generation of thromboxane A_2 and inhibited arachidonic acid-induced platelet aggregation as expected. However, this drug did not affect thrombus formation. These results indicate that occlusive thrombus formation in this model cannot be prevented solely by the prevention of thromboxane A_2 generation. Sodium ozagrel seemed to have some effect on decreasing the neurological deficit and the area of cerebral infarction, although not to a statistically significant extent.

In conclusion, we have developed a model of cerebral ischemia based on middle cerebral artery thrombosis in monkeys. The selective GPIIb/IIIa antagonist YM337 inhibited agonist-induced platelet aggregation in monkeys and this effect resulted in the prolongation of time to occlusive thrombus formation, improvement in the patency of middle cerebral artery, and a decrease in the area of cerebral infarction. GPIIb/IIIa antagonists may have therapeutic potential in the prevention of cerebral thrombosis and cerebral infarction.

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