Photothrombotic Middle Cerebral Artery Occlusion and Reperfusion Laser System in Spontaneously Hypertensive Rats

Hiroshi Yao, MD; Hiroshi Sugimori, MD; Kenji Fukuda, MD; Junichi Takada, MD; Hiroaki Ooboshi, MD; Takanari Kitazono, MD; Setsuro Ibayashi, MD; Mitsuo Iida, MD

Background and Purpose—To establish a less invasive and reproducible focal ischemia model in the rat, we adopted a 2-laser system (ie, photothrombosis and YAG laser–induced reperfusion).

Methods—The distal middle cerebral artery (MCA) of spontaneously hypertensive rats was occluded by 568-nm krypton laser and intravenous infusion of the photosensitizing dye rose bengal and was recanalized by 355-nm ultraviolet laser irradiation. Cerebral blood flow was determined by laser-Doppler flowmetry at the penumbral cortex. Infarct volume was determined at 3 days after distal MCA occlusion.

Results—Brain temperature determined with infrared thermography was maintained within an acceptable range of approximately 1°C upper shift of the center of brain temperature distribution during krypton or YAG laser irradiation. The average of the values (23 experiments; n=163) of coefficient of variation of infarct volume was 21% (coefficient of variation=30%; n=6).

Conclusions—We have characterized a highly reproducible focal ischemia model utilizing a 2-laser system, one to induce thrombotic MCA occlusion and the other to facilitate reperfusion. (Stroke. 2003;34:2716-2721.)

Key Words: cerebral ischemia, focal photochemistry reperfusion injury thrombolysis rats

To elucidate the pathophysiology of ischemic stroke and to find potential neuroprotective strategies, validated animal models of focal cerebral ischemia are indispensable. The constant objection against animal studies is the failure of pharmacological studies to translate from the animal model to the clinical setting. Nevertheless, the relevance of animal stroke models is reasonably evident, as discussed by Ginsberg. In particular, rodent models have crucial advantages of lower cost, suitability for physiological monitoring, reproducibility of lesion size, and ease of conducting replicate studies. Although Huang et al suggested that the number of animals needed to demonstrate a 50% decrease in infarction volume may be reduced by 41% in their modified transorbital baboon model compared with a previous nonhuman primate stroke model, the coefficient of variation (CV) for infarct volume was still considerably large (62%).

Tamura et al first established the proximal middle cerebral artery (MCA) occlusion model in the rat. Subsequently, distal MCA occlusion methods were developed by Chen et al and Brint et al. Recently, the intraluminal suture method has been widely used not only in rats but also in mice. However, proximal and distal MCA occlusion procedures are surgically demanding and may induce local traumatic effects, and, in the suture model, the success rate of occlusion and reproducibility of infarct size are sometimes unsatisfactory. Furthermore, the intracranial suture method is proposed to be an internal carotid artery occlusion model rather than a pure MCA occlusion model.

A photothrombotic distal MCA occlusion model in spontaneously hypertensive rats (SHR) yields a highly reproducible infarct volume and does not entail extensive surgery or opening of the dura, thereby avoiding local tissue trauma at the site of MCA occlusion. This model encompasses appropriate physiological monitoring, associated risk factors for stroke (ie, hypertension and aging), and clinically relevant pathophysiology of thrombosis. A recent immense advance in the practice of the photothrombotic stroke method in the rat achieved by Watson et al is the ultraviolet laser–induced reperfusion method. In the present study we discuss the usefulness of laser methods in establishing stroke models in SHR.

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Materials and Methods

All procedures were done in accordance with the Animal Care Guidelines at Kyushu University and the Law (No. 105) and Notification (No. 6) of the Japanese Government.

Surgical Preparation

SHR/Kyushu (aged 5 to 7 months), maintained in the Kyushu University Animal Center under specific pathogen-free conditions, were used in this study. SHR/Izm, stroke-prone SHR (SHR-SP)/Izm, Wistar-Kyoto (WKY)/Izm, and Sprague-Dawley rats were obtained from commercial vendors at the age of 3 months and used at the age of 5 to 7 months. Rats were anesthetized with halothane (3% for induction, 1.5% during the surgical preparation with a face mask, 0.75% after intubation, and 0.5% for maintenance) in a mixture of 70% nitrous oxide and 30% oxygen. The right femoral artery and vein were cannulated with the use of PE 50 tubing. The rats were endotracheally intubated with PE 240 tubing for male rats or PE 205 tubing for female rats. Pancuronium bromide (an initial dose of 0.3 mg followed by 0.1 mg every 30 minutes) was intravenously injected, and the rats were mechanically ventilated. Mean arterial blood pressure was continuously monitored. Physiological variables were maintained within normal range.

Rats were mounted on a stereotaxic head holder in the prone position, and a 2-cm incision was made vertically midway between the right orbit and the right external auditory canal. The temporalis muscle was separated and retracted, and a burr hole 3 mm in diameter was made 1 mm rostral to the anterior junction of the zygoma and squamosal bone under an operating microscope (OPMI 111, Carl Zeiss), revealing the distal segment of the MCA above the rhinal fissure. A thin bone layer was preserved to prevent injury to the brain and was carefully removed with forceps. The dura was thereby left intact.

To evaluate mechanical stress at the irradiation or occlusion site, we used 10 SHR: transient (30- or 60-minute) distal MCA occlusion by the laser method (n=2) or by a microclip (n=2) and then immediate perfusion-fixation after reperfusion; krypton and/or YAG laser irradiation without rose bengal infusion (n=3) and perfusion-fixation after 3 days of survival; and staining with TTC 3 days after either craniectomy only, clip sham occlusion, or krypton laser followed by YAG laser irradiation without rose bengal infusion (n=3).

Photothrombotic MCA Occlusion

A krypton laser operating at 568 nm (Innova 301, Coherent Inc, or 643-Y-A01, Melles Griot Inc) was used to irradiate the distal MCA at a power of 20 mW for 4 minutes. The laser beam was focused with a 30-cm focal length convex lens (KPX 112, Newport Corporation) and positioned with a mirror onto the distal MCA. The photosensitizing dye rose bengal (15 mg/mL in 0.9% saline; Wako Pure Chemical Industries Ltd) was administered intravenously at a body dose of 20 mg/kg over 90 seconds starting simultaneously with 4 minutes of laser irradiation. For permanent occlusion without corresponding counterpart groups being subjected to reperfusion of the occluded MCA, an elliptical, almost linear laser beam (linear hit)
was used to irradiate the distal MCA, as previously described.\textsuperscript{12,13} In the case of subsequent reperfusion, a round laser beam was focused at the Y-shaped juncture of the frontal and parietal branches for 2 minutes, and then the laser beam was moved to an additional site just proximal to the first irradiated site for 2 minutes (2-point hit) (Figure 1A).

**Reperfusion by YAG/Neodymium Laser Irradiation**

At certain times after MCA occlusion, a Q-switched, frequency-tripled YAG/neodymium laser operating at 355 nm (16 mW, 15 Hz, average power 2.3 W/cm\(^2\)) (Minilite II, Continuum Inc) was focused with a 30-cm focal length cylindrical lens (CKX 300, Newport Corporation) and positioned with a mirror enveloping the occluded distal MCA (Figure 1B).

**Brain Temperature Control**

Rectal and head temperatures were maintained at approximately 37.5°C and 36.5°C, respectively, by means of a warming lamp. In addition to the routine head temperature monitoring with a thermocouple probe, changes in brain temperature were determined in 2 male SHR/Kyushu with an infrared thermography system (TVS-8500, Avio System Technology Co, Ltd), in which temperature sensitivity is 0.025°C (30°C black body) and the spatial resolution is approximately 0.2 mm (Figure 2A and 2D). Brain temperature was maintained within an acceptable range of approximately 1°C upper shift of the center of brain temperature distribution during krypton or YAG laser irradiation, as shown in Figure 2.

**Measurement of Regional Cerebral Blood Flow**

In 6 male SHR/Kyushu subjected to 2 hours of transient MCA occlusion, changes in regional cerebral blood flow (CBF) were measured at 1 mm posterior and 4 mm lateral to the bregma with laser-Doppler flowmetry (ALF 21D, Advance Co Ltd). Changes in CBF were expressed as a percentage of the average of 2 or 3 baseline values. In 3 male and 5 female SHR/Kyushu, regional CBF was measured by laser-Doppler flowmetry at points 1 mm posterior and 2.0, 2.5, 3.0, 3.5, and 4.0 mm lateral to the bregma (Figure 3A) by scanning the laser-Doppler probe with a stereotaxic device before and 30 minutes after distal MCA occlusion. The distance of the infarct rim from the midline was determined on 2,3,5-triphenyltetrazolium chloride (TTC)-stained sections with National Institutes of Health Image software (version 1.56) (Figure 3B). Then the infarct rim distance was plotted against CBF, as described in Figure 3C.

**Infarct Volume**

Three days after ischemic insult, rats were decapitated under amobarbital anesthesia (100 mg/kg), and brains were rapidly removed. The brain was cooled in ice-cold saline for 10 minutes and was cut into 2-mm-thick coronal sections in a cutting block. Then the brain slices were stained with TTC (Wako Pure Chemical Industries Ltd) at 37°C for 30 minutes in the dark. Infarct volume was calculated by the trapezoidal rule with National Institutes of Health Image software (version 1.56), as previously described.\textsuperscript{13}

Eight male hypertensive rats were used for a comparative analysis between TTC and hematoxylin-eosin methods to determine the extent of infarction produced by MCA occlusion. A TTC-stained coronal slice, made at 6 mm from the frontal pole, was photographed and then stained with hematoxylin-eosin.

Reproducibility of this model was investigated by reviewing all the experiments (sham-operated or control data) done in our laboratory during 1995–2001 (Table). The surgeon-1 experiment was the first experiment for each researcher done after experiments on several (usually 5) practice rats.

**Statistical Analysis**

Values are mean±SD. The statistical differences in infarct area expressed as a percentage of hemispheric area between TTC and hematoxylin-eosin groups were determined by paired t test and linear regression analysis. Statistical power was evaluated according to Cohen.\textsuperscript{15}
Results

Effects of Mechanical Stress on Craniectomy Site

Figure 4 demonstrates the effects of craniectomy, laser irradiation, or a microclip on cortical surface morphology and MCA. Craniectomy alone produced a small necrotic lesion on the cortical surface, which was seen superior to the irradiation site (Figure 4D). On TTC-stained section, the effects of craniectomy were not remarkable, and no lesion was observed after laser irradiation (Figure 4A and 4C). However, clip sham occlusion produced a small necrotic lesion (Figure 4B) and a small amount of subarachnoid hemorrhage around the distal MCA (Figure 4F).

MCA Occlusion and Reperfusion

The irradiated MCA was completely occluded by an intraluminal thrombus within 4 minutes after simultaneous laser irradiation and rose bengal infusion, which was confirmed through the operating microscope. At approximately 1 minute of YAG laser irradiation, small streaks of blood penetrated into thrombus and gradually increased in volume, and then the entire thrombus disappeared within 3 minutes (Figure 5). In our experience, only 1 rat among a series of 26 SHR/Kyushu showed an exceptionally low major branching point of the MCA, which made YAG laser irradiation onto the entire length of occluded artery impossible.

Changes in Penumbral CBF

In 6 male SHR/Kyushu subjected to 2 hours of transient MCA occlusion, CBF was decreased to 32±16% of the control values after distal MCA occlusion for 10 minutes and was stable thereafter. After YAG laser–induced reperfusion, CBF increased to 98±21%. Infarct volume in these rats was 61±18 mm³ (CV=30%).

In 1 male SHR/Kyushu subjected to permanent MCA occlusion, the distance of the infarct rim from midline was 3.1 mm (Figure 3B), which indicated that the flow threshold for infarction was 41% of the resting CBF (Figure 3C). Male SHR/Kyushu showed a flow threshold of 43±5% of the resting CBF. In 5 female SHR/Kyushu, the flow threshold for infarction was calculated as 32±5% of the resting CBF.

Reliability and Reproducibility of Infarct Volume

The size of infarct area was 23.1±1.9% on TTC-stained slices, which was not different from the value of 23.8±2.5% on hematoxylin–eosin–stained sections (P=0.207, paired t test). Linear regression analysis revealed a good correlation between the 2 methods, where r=0.816, slope=1.054, and y intercept=−0.550%, which is significantly different from a line with slope=0 (P=0.0134) and is not significantly different from a y intercept=0 (P=0.9404). Thus, TTC staining was a reliable indicator of 3-day-old infarction.

An average of the values of CV of infarct volume was 21±6%, indicating high reproducibility for this model (Table). Therefore, in case of the 30% differences in infarct volume between the groups (eg, 30% reduction in infarct volume after treatment), the effect size d is 1.43 (30%/21%). The relative seriousness of type I to type II error is considered to be 0.05/0.20. Then, from Table 2.4.1 by Cohen,15 for significance criterion a2=0.05 for 2-tailed test, effect size d=1.40, and row power d=0.80, the number of 9 rats in each group is enough to exclude type II error.

Discussion

We adopted the photothrombotic strategy instead of mechanical occlusion of the MCA because the former does not entail opening the dura. Although any model requiring craniectomy necessitates a good deal of skill, the advantages of this method include relatively slight invasiveness, negligible incidence of mortality, and a high degree of reproducibility. In case of mechanical occlusion, for example, an unexpected finding of Glazier et al16 was cortical expression of heat shock protein 72 and induction of ischemic tolerance after sham occlusion of the distal MCA, indicating substantial stress to the cortex even in sham-occlusion rats. Scanning electron micrography of luminal MCA showed that the irradiated segment of the MCA...
appeared relatively normal, whereas the endothelial layer was damaged at the clipped site.\textsuperscript{17,18} Although the suture method has the advantage of not requiring craniectomy with its associated surgical trauma, mortality was high in some studies,\textsuperscript{19} or the survival time was short, and subarachnoid hemorrhage may occur because of perforation of intracranial arteries.\textsuperscript{20}

Koizumi’s method of MCA occlusion by intraluminal suture,\textsuperscript{6} subsequently modified by Longa et al,\textsuperscript{7} gained popularity in stroke research owing to the relative simplicity of the surgical procedure. By coating the suture with poly-L-lysine, a polycationic substance thought to make the suture more adherent to the vascular endothelium, Belayev et al\textsuperscript{10} showed a much more consistent infarction (CV=38\% and 8\%). In our photothrombotic MCA occlusion model, all rats develop infarction after krypton laser irradiation and rose bengal infusion with highly reproducible infarct volumes (average CV=21\%). This photothrombotic model permits a reduction in the number of animals needed to establish the efficacy of a therapeutic agent.

The basis of this photothrombosis model is functional endothelial damage stimulated by an intravascular photochemical reaction, which results in a specific platelet-based response to cerebral vessel damage.\textsuperscript{20} Pathological phenomena unique to thrombotic stroke have been observed: thrombogenically activated blood leads to the formation of blood-borne factors, which causes detrimental effects on blood-brain barrier and on ischemic neurons.

Coefficient of variation (CV) = SD/mean. M, male; F, female
*Not subjected to reperfusion.
†Female aged SHR/Kyushu.
‡2-point hit.

Reproducibility of Infarct Size

<table>
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<tr>
<th>Surgeon</th>
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<td>M</td>
<td>82±18</td>
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Figure 4. A to C, TTC-stained section of craniectomy only (A), clip sham occlusion (B), and krypton laser irradiation (20 mW, 4 minutes) followed by YAG laser irradiation (16 mW, 3 minutes). Bar=2 mm. B, Clip sham occlusion caused a necrotic lesion (bracket). D, Hematoxylin-eosin–stained coronal section shows a necrotic lesion (arrowheads) by craniectomy. Bracket indicates irradiation site; RF, rhinal fissure. Bar=1 mm. E and F, Distal MCA photochemically occluded for 30 minutes followed by YAG laser–induced reperfusion (E) or occluded by a microclip for 30 minutes (F). F, A small amount of subarachnoid hemorrhage around the distal MCA was observed (asterisks). Bar=100 μm.

Figure 5. A, Distal MCA before photothrombotic occlusion. Bar=1 mm. B, Occluded distal MCA (bracket). C, At 1 minute of YAG laser irradiation, the occluded distal MCA was partially reperfused. D, Reperfused distal MCA.
mic brain. Therefore, this photochemically induced MCA occlusion model would facilitate greater understanding of the pathophysiology of thrombotic stroke.

The mechanisms of ultraviolet laser-induced–vascular dilatation are attributed to rapidly expanding vapor bubble formed intraluminally in smooth muscle at high energy density or photolytic release of nitric oxide at low energy density. Ultraviolet laser–induced dilatation facilitated formation of the microscopic intrathrombus channels that lead to recanalization of a platelet-occluded artery. The early reperfusion apparently confers advantages on ischemic brain tissue at risk, yet the return of blood flow in the postischemic brain has a negative side. For instance, a late secondary drop in the apparent diffusion constant was observed in some ischemic tissues of both rats and humans that appeared initially to be salvaged by reperfusion therapy. Reperfusion injury may be possibly related to this secondary deterioration. Hence, the therapeutic time window for reperfusion may be prolonged when combined with treatments that counteract reperfusion injury. Antagonists of various factors such as excitotoxicity, spectrin breakdown, free radicals, or apoptosis may be desirable in combination with reperfusion therapy.

In conclusion, we have characterized our SHR stroke model utilizing a 2-laser system, one to induce cerebral arterial occlusion and the other to facilitate its elimination. This model fulfills the standard of an acceptable focal ischemia model (ie, highly reproducible infarct volume) with a less invasive surgical procedure.

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