

TABLE 2: Prevalence and grade of microbleeds in patients with primary stroke

Stroke subtype	Microbleeds n (%)	Grade of Microbleeds			
		Absent (grade 0), n	Mild (grade 1), n	Moderate (grade 2), n	Severe (grade 3), n
Atherothrombotic (n = 22)	5 (22.7)	17	3	2	0
Cardioembolic (n = 13)	0 (0)	13	0	0	0
Lacunar (n = 31)	7 (22.6)	24	4	0	3
Intracerebral hemorrhage (n = 36)	17 (47.2)	19	5	10	2
Total (n = 102)	29 (28.4)	73	12	12	5

TABLE 3: Combination of stroke subtypes in patients with recurrent stroke

Combination of stroke subtype*	Antiplatelet or Anticoagulation Therapy after Previous Stroke	Patients, n	Microbleeds, n
Intracerebral hemorrhage/intracerebral hemorrhage		9	8
Lacunar/lacunar	+	6	2
Intracerebral hemorrhage/lacunar		9	8
Intracerebral hemorrhage (three times)		4	4
Lacunar/intracerebral hemorrhage	-	3	2
Atherothrombotic/lacunar	+	3	1
Intracerebral hemorrhage/atherothrombotic		2	1
Atherothrombotic/atherothrombotic	-	2	1
Lacunar/atherothrombotic	-	2	2
Lacunar/lacunar	-	2	1
Lacunar/atherothrombotic	+	2	1
Lacunar/intracerebral hemorrhage	+	2	2
Cardioembolic/cardioembolic	+	2	1
Atherothrombotic/atherothrombotic	+	2	2
Intracerebral hemorrhage/cardioembolic		1	0
Lacunar (three times)	+	1	1
Atherothrombotic/lacunar	-	1	0
Cardioembolic/cardioembolic	-	1	0
Total		54	37

\* Combination of stroke subtype expressed as previous stroke subtype/latest stroke subtype; Present is indicated by + and absent by -.

Prevalences and grades of microbleeds in the patients in the primary stroke group are summarized in Table 2. Microbleeds were observed more frequently in patients with intracerebral hemorrhage (47.2% of the patients) than in patients with atherothrombotic infarction (22.7%) or lacunar infarction (22.6%). Microbleeds were not observed in patients with cardioembolic infarction. The grade of microbleeds was highest in patients with intracerebral hemorrhage, with statistical significance for patients with cardioembolic infarction ( $P < .05$ ). A significant correlation was shown between grade of microbleeds and severity of leukoaraiosis ( $r = 0.367$ ,  $P < .001$ ). No significant difference was shown in incidences of intracranial large-artery diseases between patients with primary stroke with microbleeds and patients with primary stroke without microbleeds.

Combinations of stroke subtypes in patients with recurrent stroke are summarized in Table 3. Two patients with intracerebral hemorrhage who had undergone antiplatelet therapy after previous ischemic stroke (cases of lacunar/intracerebral hemorrhage) exhibited severe microbleeds. On the other hand, three patients who had not undergone antiplatelet or

anticoagulant therapy after previous ischemic stroke exhibited intracerebral hemorrhage (cases of lacunar/intracerebral hemorrhage), and two of the three exhibited microbleeds. Patients with recurrent stroke had a significantly higher prevalence of microbleeds than did patients with primary stroke (68.5% versus 28.4%,  $P < .0001$ ). The grade of microbleeds was also significantly higher in patients with recurrent stroke than in patients with primary stroke ( $P < .0001$ ). Representative T2- and T2\*-weighted MR images are shown in Figure 1. The prevalences and grades of microbleeds in patients with ischemic stroke (including atherothrombotic infarction, cardioembolic infarction, and lacunar infarction) alone, in patients with intracerebral hemorrhage alone, and in patients with a combination of ischemic stroke and intracerebral hemorrhage are summarized in Table 4. Frequency of microbleeds was highest in the group of patients with intracerebral hemorrhage alone (92.3%), next highest in the group of patients with intracerebral hemorrhage and ischemic stroke (76.5%), and then in the group of patients with ischemic stroke alone (50.0%) ( $P < .05$ ). The order of grades of microbleeds was the same: highest in the

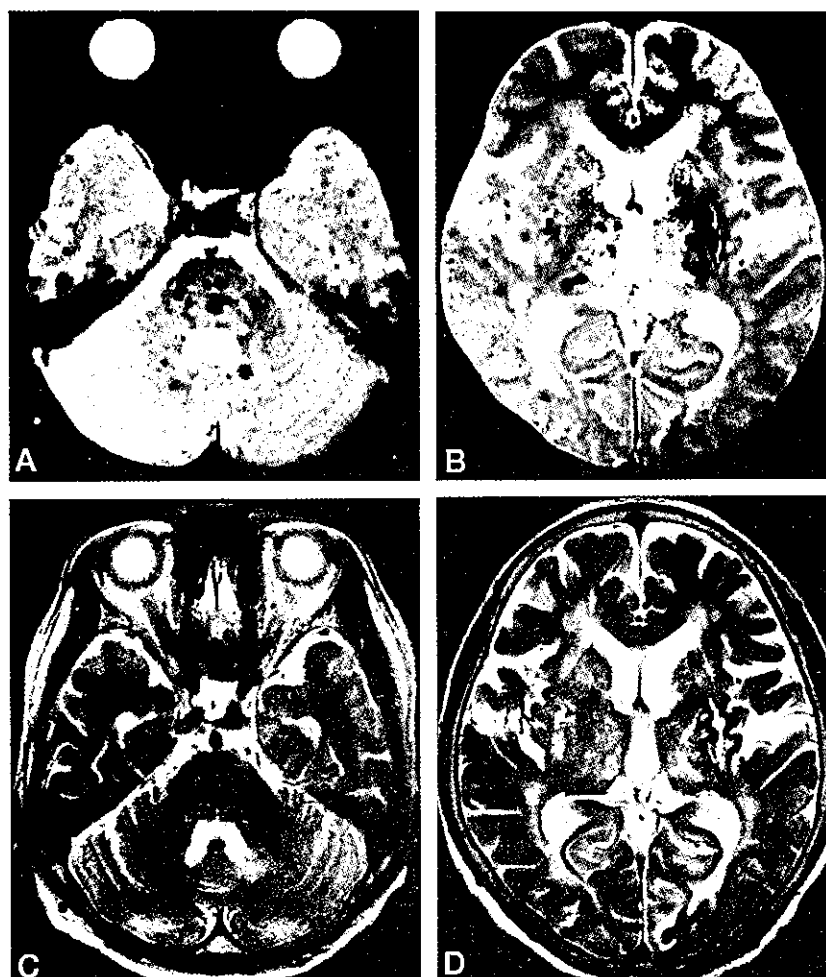


FIG 1. MR images of a 75-year-old patient with intracerebral hemorrhage in the right parietal lobe 8 years after the occurrence of intracerebral hemorrhage in the left putamen.

A and B, T2\*-weighted gradient-echo images (800/26; flip angle, 20 degrees) reveal multiple foci of signal intensity loss (microbleeds) in the brain stem, cerebellum, basal ganglia, and cerebral hemispheres. In addition, old intracerebral hemorrhage is evident in the left putamen.

C and D, T2-weighted spin-echo images (4500/112) show the site of old intracerebral hemorrhage in the left putamen, but microbleeds are not evident.

TABLE 4: Prevalence and grade of microbleeds in patients with each combination of subtypes of recurrent stroke

Combination of stroke subtype	Microbleeds n (%)	Grade of Microbleeds			
		Absent (grade 0), n	Mild (grade 1), n	Moderate (grade 2), n	Severe (grade 3), n
Intracerebral hemorrhage alone (n = 13)	12 (92.3)	1	1	4	7
Intracerebral hemorrhage and ischemic stroke (n = 17)	13 (76.5)	4	1	5	7
Ischemic stroke alone (n = 24)	12 (50.0)	12	4	5	3
Total (n = 54)	37 (68.5)	17	6	14	17

group of patients with intracerebral hemorrhage alone, next highest in the group of patients with intracerebral hemorrhage and ischemic stroke, and then in the group of patients with ischemic stroke alone ( $P < .01$ ). Although a significant correlation was found between grade of microbleeds and severity of leukoaraiosis in the patients with recurrent stroke ( $r = 0.553$ ,  $P < .0001$ ), no significant difference was observed in incidences of intracranial large-artery diseases between patients with recurrent stroke with microbleeds and patients with recurrent stroke without microbleeds.

Among the patients with intracranial large-artery diseases (54 patients), 22 were shown to have micro-

bleeds. Patients with intracranial large-artery diseases and microbleeds showed a significantly higher prevalence of hypertension or lacunar infarction (90.9%) than did those with intracranial large-artery diseases but without microbleeds (59.4%) ( $P = .0110$ ).

Results of logistic regression analysis showed that recurrent stroke (odds ratio, 4.487; 95% confidence interval, 1.989–10.120) and leukoaraiosis (odds ratio, 5.079; 95% confidence interval, 2.125–12.143) were significantly and independently associated with presence of microbleeds, whereas atrial fibrillation had a significant and independent negative association with presence of microbleeds (Table 5).

TABLE 5: Logistic regression analysis

Variable	Odds Ratio	95% CI	P
Age	0.992	0.957–1.029	.6771
Sex	1.764	0.754–4.125	.1905
Hypertension	1.986	0.823–4.794	.1271
Atrial fibrillation	0.209	0.047–0.934	.0404
Diabetes mellitus	1.257	0.495–3.192	.6306
Hyperlipidemia	0.566	0.205–1.564	.2721
Intracranial large-artery diseases	0.981	0.425–2.264	.9642
Leukoaraiosis	5.079	2.125–12.143	.0003
Recurrent stroke	4.487	1.989–10.120	.0003

Note.—CI indicates confidence interval.

### Discussion

In the present study, we evaluated frequency of microbleeds in patients with recurrent stroke and association of microbleeds with combination of stroke subtypes and severity of leukoaraiosis. The following results were obtained: 1) prevalence and grade of microbleeds were significantly higher in patients with recurrent stroke than in patients with primary stroke; 2) microbleeds were observed more frequently and were of higher grade in patients with intracerebral hemorrhage than in patients with either primary or recurrent ischemic stroke but were frequently observed even in patients with recurrent ischemic stroke (prevalence of 50%); and 3) leukoaraiosis was more severe in patients with recurrent stroke than in patients with primary stroke, and a correlation was found between grade of microbleeds and severity of leukoaraiosis in both patients with primary stroke and those with recurrent stroke.

Previous studies using T2\*-weighted MR imaging have revealed that there is an association between presence of microbleeds and small-artery disease (3–10), as was also indicated by the results of the present study, which showed a high frequency of microbleeds in patients with intracerebral hemorrhage or severe leukoaraiosis but no association between presence of microbleeds and intracranial large-artery diseases. Association of microbleeds with small-artery diseases but not with intracranial large-artery diseases was further indicated by the results; among patients with intracranial large-artery diseases, those with microbleeds showed a significantly higher prevalence of hypertension or lacunar infarction, which is associated with small-artery disease, than did those without microbleeds. However, to the best of our knowledge, few studies have focused on microbleeds in patients with recurrent stroke. Some studies have included cases of recurrent stroke. Roob et al (5) reported that among patients with primary intracerebral hemorrhage, those with microbleeds more frequently have histories of stroke than do those without. Kato et al (7) revealed a correlation between number of microbleeds and number of intracerebral hemorrhages or lacunar infarctions. However, no detailed evaluation of the combination of stroke subtypes was conducted in those studies.

The present study clearly showed that patients with recurrent stroke had a higher prevalence and higher

grade of microbleeds than did those with primary stroke. In addition, the prevalences of microbleeds were similar but not exactly the same in patients with primary and recurrent stroke with each subtype of stroke; although microbleeds were associated more with intracerebral hemorrhage than with ischemic stroke in patients with primary and recurrent stroke, their prevalence was high in patients with recurrent stroke with all combinations of subtypes. Association between microbleeds and symptomatic intracerebral hemorrhage has been shown in many studies (8–10, 20–24). Tsushina et al (9) reported that the presence of microbleeds was most significantly correlated with history of hemorrhagic stroke. Microbleeds have been reported to be a risk factor for acute postischemic cerebral hemorrhage (22, 23) and also to be associated with intracerebral hemorrhage in patients with cerebral amyloid angiopathy (20, 21). In addition, some studies have indicated that the presence of microbleeds increases the risk of hemorrhagic transformation in patients receiving thrombolytic therapy for acute ischemic stroke (23) or the risk of aspirin-associated intracerebral hemorrhages (10). In the present study, two antiplatelet-treated cases of intracerebral hemorrhage exhibited severe microbleeds. The presence of microbleeds may identify the patients with bleeding-prone microangiopathy, by which decision of using antiplatelet or anticoagulation therapy after ischemic stroke would be possible. Cohort studies are needed to clarify whether patients with microbleeds are really prone to bleeding or whether both microbleeds and symptomatic intracerebral hemorrhage occur after the progression of microangiopathy.

The notable findings of this study are the high frequency of microbleeds in patients with recurrent stroke and the different microbleed prevalences in patients with each subtype of recurrent stroke than in patients with each subtype of primary stroke. Not only were extremely high prevalences shown in patients with intracerebral hemorrhage alone and in patients with a combination of intracerebral hemorrhage and ischemic stroke, but also, a relatively high prevalence (50%) was shown in patients with ischemic stroke alone. This may be explained by the more severe leukoaraiosis in patients with recurrent stroke than in patients with primary stroke. Previous studies have revealed that microbleeds are associated with leukoaraiosis (3, 5, 7–9) but not with intracranial large-artery diseases (18), indicating that small-artery disease results in the presence of microbleeds. In addition, leukoaraiosis has been shown to be closely linked with both intracerebral hemorrhage and ischemic injury (25–27). Either rupture or occlusion associated with microangiopathy may result in intracerebral hemorrhage or ischemic stroke, depending on the circumstances. Kim et al (8) revealed that microbleeds are a predictor of intracerebral hemorrhage in patients with no or mild leukoaraiosis but they occur similarly in association with both ischemic stroke and hemorrhagic stroke in patients with advanced leukoaraiosis. Progression of leukoaraiosis in the course of

continuous disturbance of a small artery related to long-standing exposure to stroke risk factors after primary stroke might result in the appearance of microbleeds and finally result in recurrence of stroke of any subtype, not restricted to intracerebral hemorrhage.

### Conclusion

Asymptomatic microbleeds shown by T2\*-weighted MR imaging frequently occur in patients with recurrent stroke, either hemorrhagic or ischemic stroke, and the microbleeds are closely associated with severity of leukoaraiosis. The presence of microbleeds may be an increased risk factor for recurrent stroke, and we might be able to identify those patients who are likely to experience recurrent stroke.

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## Clinical significance of cerebrovascular reserve in acetazolamide challenge —Comparison with acetazolamide challenge H<sub>2</sub>O-PET and Gas-PET—

Masao IMAIZUMI,\* Kazuo KITAGAWA,\*\* Naohiko OKU,\* Kazuo HASHIKAWA,\*\* Masashi TAKASAWA,\*\*  
Takuya YOSHIKAWA,\*\* Yasuhiro OSAKI,\* Kouji MATSUSHITA,\*\* Masayasu MATSUMOTO,\*\*  
Masatsugu HORI\*\* and Jun HATAZAWA\*

\*Division of Nuclear Medicine and Tracer Kinetics, Osaka University Graduate School of Medicine

\*\*Department of Internal Medicine and Therapeutics, Osaka University Graduate School of Medicine

**Objective:** The response of cerebral blood flow (CBF) to acetazolamide (ACZ) challenge is frequently determined in clinical settings to evaluate cerebrovascular reserve (CVR). A reduced CVR can indicate patients with occlusive cerebrovascular disease and compromised hemodynamics who may be at increased risk of cerebral ischemia. However, how precisely ACZ reflects cerebral hemodynamic impairment remains obscure. The present study aims to clarify the pathological significance of CVR in patients with occluded carotid arteries. **Methods:** We recruited seventeen patients with occlusive lesions in the internal carotid artery (ICA) or middle cerebral artery (MCA). We assessed these patients in terms of resting cerebral blood flow (CBF) and the CVR response to ACZ challenge using H<sub>2</sub>O positron emission tomography (PET). In addition, we evaluated hemodynamic parameters including oxygen extraction fraction (OEF) using Gas-PET. **Results:** We identified a significant negative correlation between the CVR and OEF or the cerebral blood volume (CBV)/CBF ratio, as a potential index of cerebral perfusion pressure. Although the CVR values were reduced in all regions with elevated OEF (Stage II), these values were highly variable regardless of the CBV/CBF ratios. The cut-off value of CVR alone could not detect Stage II, but when combined with resting CBF, misery perfusion accompanied by increased OEF was detected with high sensitivity (6/7) and specificity (61/62). **Conclusion:** CVR could be applied as an index reflecting both autoregulatory capacity and OEF. The present study also supported the notion that SPECT with ACZ challenge can be clinically applied to detect misery perfusion.

**Key words:** acetazolamide, hemodynamics, positron emission tomography

### INTRODUCTION

DETERMINING THE DEGREE of hemodynamic compromise in chronic cerebrovascular disease is clinically important to predict subsequent ischemic stroke.<sup>1–3</sup> According to Powers' classification of chronic hemodynamics compromised with occlusive cerebrovascular disease,<sup>4,5</sup> hemo-

dynamic impairment can be categorized into two stages. Stage I (autoregulatory vasodilatation) is defined as an increase in cerebral blood volume (CBV) in the hemisphere distal to the occlusive lesion, with normal cerebral blood flow (CBF), oxygen extraction fraction (OEF), and cerebral metabolism rate for oxygen (CMRO<sub>2</sub>). Stage II (autoregulatory failure) is characterized by reduced CBF and increased OEF with normal CMRO<sub>2</sub> and is termed "misery perfusion."<sup>6</sup> These stages were originally defined using <sup>15</sup>O gas steady state positron emission tomography (Gas-PET) and have been widely applied to assess patients with severe atherosclerotic carotid artery stenosis or occlusion. Although PET is a very reliable quantitative tool with which to evaluate cerebral hemodynamic status, the procedure is expensive and is not routinely available.

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For reprint contact: Masao Imaizumi, M.D., Ph.D., Division of Nuclear Medicine and Tracer Kinetics, Osaka University Graduate School of Medicine, 2-2, Yamadaoka, Suita, Osaka 565-0871, JAPAN.

E-mail: imaizumi@tracer.med.osaka-u.ac.jp

Cerebral blood flow can be measured quantitatively or qualitatively using several methods, including single photon emission computed tomography (SPECT). However, since individual measurements of CBF alone do not adequately assess cerebral hemodynamic status, paired blood flow measurements are taken with resting values being established, followed by measurements after stimulation of cerebral vasodilation. The CBF response to acetazolamide (ACZ) challenge determined as cerebrovascular reserve (CVR) in SPECT, transcranial Doppler (TCD), and magnetic resonance angiography (MRA), is frequently used as an index of autoregulatory vasodilation in clinical settings to evaluate hemodynamic impairment. However, relationships between CVR measured by PET and other hemodynamic parameters must be clarified before CVR can be widely used as an index of cerebral hemodynamic impairment. The present study attempts to establish the CVR to ACZ as a reliable index of Stage I and Stage II hemodynamic compromise in patients with chronic occlusive cerebrovascular disease using both Gas-PET and H<sub>2</sub>O-PET.

## MATERIALS AND METHODS

### Patients

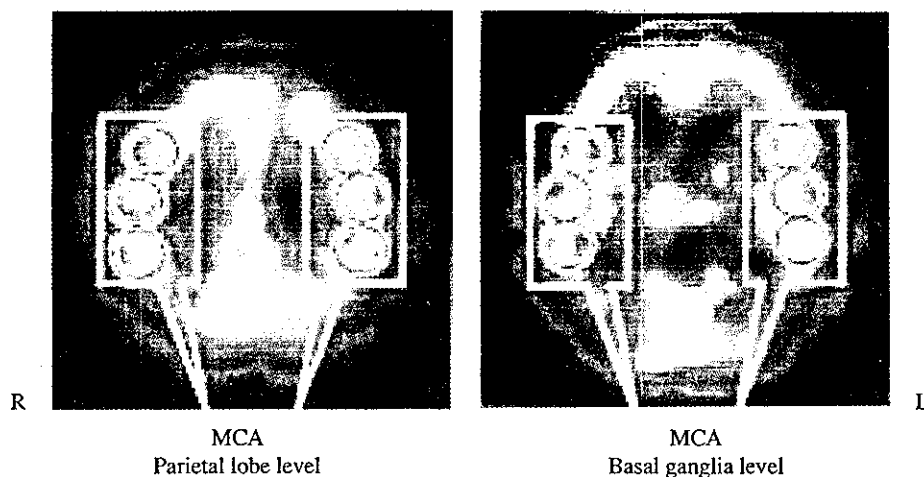
We used simultaneous Gas-PET and ACZ challenge H<sub>2</sub>O-PET to examine 25 consecutive patients with cerebrovascular disease at Osaka University Medical School Hospital. Immediately before the PET study, each patient underwent neurological and neuro-radiological evaluations, including an evaluation for occlusive cerebrovascular disease by Doppler ultrasonography, MRI, magnetic

resonance angiography (MRA), and cerebral angiography. MRI was performed in the orbito-meatal plane with 5-mm-thick sections using a 1.5-T unit (1.5-T Sigma Horizon; GE Medical System; 1.5-T Magnetome Vision; Siemens). Infarction was defined as a focal area with prolonged T1 and T2 relaxation times. The maximal interval between the MRI and PET studies was 30 days. Patients underwent the PET study at least 4 weeks after their most recent clinical episode, when their neurological condition had stabilized. Cerebral angiography was performed in all patients. The maximum percentage stenosis and the presence of ulceration, which can cause artery-to-artery embolism, were evaluated according to the recommendations of the North American Symptomatic Carotid Endarterectomy Trial (NACET).<sup>7</sup> We studied severe stenosis (70%–99%) and cerebrovascular artery occlusion. And, the mechanism of stroke in each patient was clinically diagnosed and classified according to the National Institute of Neurological Disorders and Stroke (NINDS) Classification of cerebrovascular disease III.<sup>8</sup> Patients with embolic infarction (large infarction) who had cardioembolic risk factors such as atrial fibrillation, valvular heart disease, or myocardial infarction and with acute phase were excluded from this study. We finally studied 17 consecutive patients [8 men, 9 women; mean age 56.5 ± 16.7 yr (mean ± SD)]. Five patients had cerebral infarction (lacuna infarction and/or minimal infarction in the territory of the affected arteries), eight had transient ischemic attacks, and four had asymptomatic carotid artery disease. Table 1 summarizes the clinical features and the angiographic and MRI findings.

Table 1 Patient characteristics

Patient No.	Age/Sex	Neurological Deficits	Disease	Angiographic Findings	MRI Findings
1	31/F	Rt hemiparesis	TIA	Bil ICAO	Rt BG*
2	71/M	Lt hemiparesis	TIA	Rt ICAO	Bil BG*
3	59/M	Headache	TIA	Rt MCAS	Bil BG & CR*
4	66/M	Rt hemiparesis & Dysarthria	CI	Lt MCAO	Lt MCA territory**
5	29/F	Lt hemiparesis & Visual field defect	CI	Bil ICAO	Rt parieto-occipital lobe**
6	62/M	Rt sensory disturbance	CI	Lt ICAS	Bil BG*
7	69/F	Lt hemiparesis	TIA	Rt MCAO	None
8	19/M	None	Asymptomatic	Bil MCAO	Lt parietal lobe**
9	39/F	Lt hemiparesis & Dysarthria	TIA	Rt MCAO	None
10	67/M	Dizziness	Asymptomatic	Lt ICAO	Lt BG*
11	74/M	Lt ischemic retinopathy	Asymptomatic	Lt ICAO	None
12	72/M	None	Asymptomatic	Lt ICAS	Lt CR*
13	63/F	None	TIA	Lt ICAS	None
14	56/F	Lt hemiparesis	TIA	Rt MCAO	Rt MCA territory**
15	59/F	Dysarthria	CI	Bil ICAO	Lt CR*
16	69/F	Lt sensory disturbance	CI	Rt MCAO	Rt CR*
17	56/F	Lt hemiparesis	TIA	Rt ICAS	None

Rt, right; Lt, left; Bil, bilateral; TIA, transient ischemic attack; CI, cerebral infarction; Asymptomatic; asymptomatic carotid artery disease; MCA, middle cerebral artery; MCAS, middle cerebral artery stenosis; MCAO, middle cerebral artery occlusion; ICAS, internal carotid artery stenosis; ICAO, internal carotid artery occlusion; BG, basal ganglia; CR, corona radiata; \*lacuna infarction; \*\*small infarction (<15 mm)



**Fig. 1** Comparison of CVR and gas-PET parameters. A comparison of CVR and gas-PET parameters in 68 ROI in all MCA territories revealed significant negative correlations between CVR and OEF ( $r = -0.559$ ;  $p < 0.0001$ ) and between CVR and CBV/CBF ( $r = -0.331$ ;  $p < 0.0095$ ). Values of CBF and  $CMRO_2$  were not correlated with CVR.

### PET Imaging

All patients were scanned with a Headtome V/SET 2400W system (Shimadzu Co., Ltd., Kyoto, Japan), which acquires 63 slices with an interslice distance of 3.1 mm. All scans were performed at a resolution of 3.7 mm FWHM in the transaxial direction and at 5 mm in the axial direction. The patient's head was immobilized in a holder and positioned using light beams to obtain transaxial slices parallel to the orbito-meatal line. Before the PET study, we performed germanium 68-gallium 68 transmission scanning over 10 minutes for attenuation correction. Images were reconstructed using an ordered-subset expectation maximization (OS-EM) algorithm (12 iterations with 4 ordered subsets). The [ $^{15}O$ ]gas steady state method required the patients to inhale a mixture of  $C^{15}O_2$  (550 MBq/min) and  $^{15}O_2$  (1,300 MBq/min) through a mask. The scan time was nine minutes, and four samples of blood were manually obtained from the radial artery during each scan. We measured the concentration of the radiotracer activity in whole blood and plasma using a well counter, as well as the arterial blood hematocrit, hemoglobin concentration,  $PaO_2$  and  $PaCO_2$  values. The CBV was measured after inhalation of 2,000 MBq of  $C^{15}O$  and a 9-minute scanning period. We manually sampled arterial blood three times during the scan and measured radiotracer activity in whole blood. The values for CBF,  $CMRO_2$ , and OEF were calculated based on the steady state method, and  $CMRO_2$  and OEF were corrected according to the CBV.

After the Gas-PET examination, patients received a 36-sec intravenous bolus of 1,110 MBq  $H_2^{15}O$  at a flow rate of 30 ml/min through a cannula placed in the antecubital vein to initiate ACZ challenge [ $^{15}O$ ]H $_2$ O PET. Data were acquired over a scanning period of 160 s using a  $128 \times 128$  matrix. Regional CBF was determined from the  $H_2^{15}O$

bolus injection and autoradiographic methods while the participants were in a resting state and 10 minutes after the injection of ACZ. Input function was evaluated for 4 minutes at a rate of 5 ml/min by continuous arterial blood sampling via a catheter needle inserted in the radial artery, and  $^{15}O$  radioactivity was concurrently measured using a beta-detector (Shimadzu Corp., Kyoto, Japan). The study protocol complied with the standard ethical guidelines of Osaka University Medical School, and written informed consent was obtained from all participants.

### Data Analysis

All PET data were analyzed using the "Dr. View pro5.0" image analysis software system (Asahi Kasei Joho System Co., Ltd., Tokyo, Japan) running on a UNIX system and an Indigo 2 station (Silicon Graphics, Mountain View, CA, USA). Circular regions-of-interest (ROI), 20 mm in diameter, were placed over the cortex at the levels of the basal ganglia (lower MCA territory) and parietal lobe (upper MCA territory) on PET images from each patient (Fig. 1). All ROI generated on the resting CBF and ACZ-challenge CBF images measured using  $H_2O$ -PET were transferred to the CBF, OEF,  $CMRO_2$ , and CBV images measured using Gas-PET. All ROI on PET images can be made to exactly coincide using this system. Sixty-eight regions were finally investigated in images from 17 patients (four regions per patient: right, left, upper and lower MCA). The following equation estimated the percentage increase in rCBF induced by the ACZ challenge in the form of the CVR:  $CVR = (ACZ \text{ challenge CBF} - \text{Resting CBF}) / \text{Resting CBF}$ .

Seven age-matched normal volunteers with non-focal neurological symptoms and without evidence of ischemic lesions after MRI or stenotic lesions in the major cerebral arteries after MRA, underwent  $H_2O$ -PET to determine

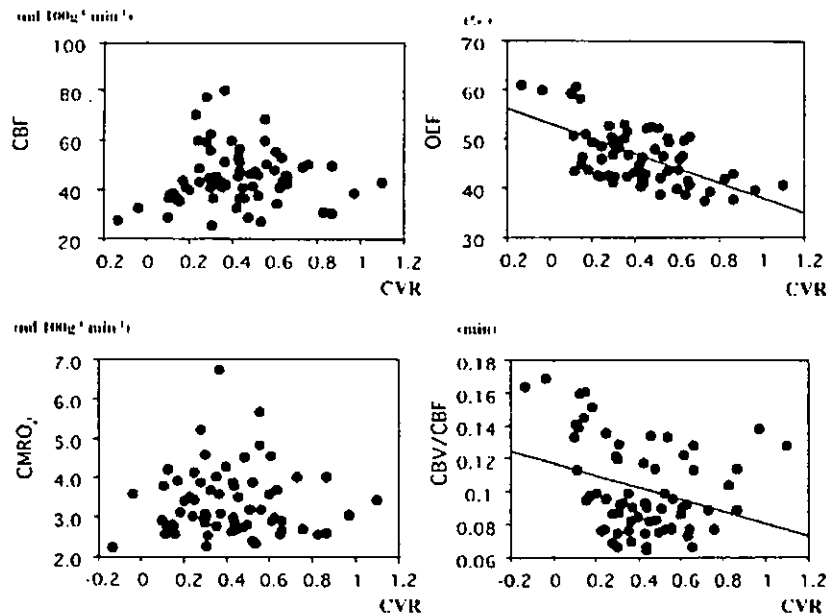


Fig. 2 ROI setting. Circular regions-of-interest (ROIs), 20 mm in diameter, were placed on cerebral cortices of middle cerebral artery territories on the levels of basal ganglia and parietal lobe in PET images of each patient.

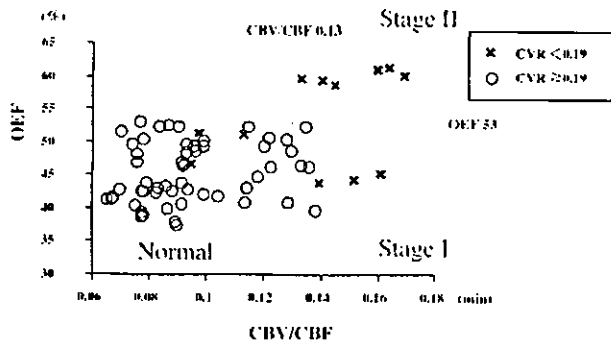


Fig. 3 CVR measured by ACZ challenge H<sub>2</sub>O-PET in normal, Stage I and Stage II regions. Plot of values of OEF versus CBV/CBF ratio for all 68 ROI with complete quantitative PET studies. Abnormal values (mean + 2SD) for OEF and CBV/CBF ratio are indicated by dotted lines. All six regions diagnosed as Stage II by Gas-PET (upper right quadrant) corresponded perfectly with reduced CVR (CVR < 0.19). CVR values were decreased in three of 54 regions diagnosed as normal (lower left quadrant) and in three of eight regions diagnosed as Stage I (lower right quadrant).

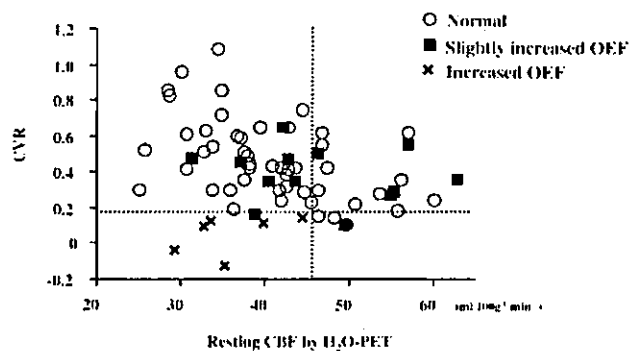
normal control values. Normal Gas-PET values from healthy controls were not available. We therefore utilized Gas-PET parameter values from seven patients with no infarction and no severe stenosis or occlusion (<50%) who had non-specific brain symptoms without focal signs (pre-operation for cerebral aneurysm, headache, dizziness and syncope) as the normal values. We assessed the relationship between the CVR measured by H<sub>2</sub>O PET and

the Gas-PET parameters in the MCA territories using linear-regression analysis and Pearson's correlation coefficient. All data are expressed as means  $\pm$  SD. Differences in data between groups were statistically evaluated using an unpaired t-test. Differences with p values of <0.05 were considered statistically significant.

## RESULTS

The normal control values for the CBF and CVR measured by H<sub>2</sub>O PET for the MCA territories were  $45.5 \pm 8.5$  ml/100 g<sup>-1</sup> min<sup>-1</sup> and  $0.49 \pm 0.15$  (mean  $\pm$  SD). The CVR values were judged abnormal when beyond the mean - 2SD of the normal controls. All 68 regions were classified into two groups according to the CVR values measured by H<sub>2</sub>O PET: reduced group (N = 12) with a CVR value of <0.19 (mean - 2SD) and a normal group (N = 56) with a CVR of 0.19 or higher. We used normal Gas-PET parameter values as follows: CBF,  $46.9 \pm 11.3$  ml/100 g<sup>-1</sup> min<sup>-1</sup>; OEF,  $44.1 \pm 4.62\%$ ; CMRO<sub>2</sub>,  $3.39 \pm 0.82$  ml/100 g<sup>-1</sup> min<sup>-1</sup>; CBV,  $4.22 \pm 0.75$  ml/100 g<sup>-1</sup>; and CBV/CBF ratio,  $0.09 \pm 0.02$  min. All regions were classified into three groups based on their OEF values: normal, OEF < 48.7% (mean + 1SD of the mean OEF value); slightly increased,  $48.7\% \leq$  OEF < 53.3% (mean + 2SD of the mean OEF value) and increased, OEF  $\geq$  53.3%. We classified 68 regions into three groups compared with the base of our normal ranges of CBV/CBF and OEF. The three groups were normal [CBV/CBF < 0.13 min (mean + 2SD of the mean CBV/CBF ratio); OEF < 53.3%; N = 54], Stage I [CBV/CBF  $\geq$  0.13 min and OEF < 53.3%; N





**Fig. 4** Detection of Stage II cerebral hemodynamic failure using ACZ challenge H<sub>2</sub>O-PET. Plot of CVR versus resting CBF values for all 68 ROI with quantitative ACZ challenge H<sub>2</sub>O-PET studies. Cut-off values for CVR and CBF of 0.19 (mean - 2SD) and 46.9 ml 100 g<sup>-1</sup> min<sup>-1</sup> (normal value) respectively (*lower left quadrant*), led to sensitivity and specificity values of 86% (6/7) and 98% (61/62), respectively, for detecting misery perfusion (OEF ≥ 53.3%).

= 8] and Stage II [OEF ≥ 53.3%; N = 6].

#### Comparison of CVR with other PET parameters

We compared the CVR and PET parameters in the 68 ROI of all MCA territories and found a significant negative correlation between CVR and OEF ( $r = -0.559$ ;  $p < 0.0001$ ; Fig. 2) and between CVR and CBV/CBF ( $r = -0.331$ ;  $p < 0.0095$ ; Fig. 2). The CBF and CMRO<sub>2</sub> did not correlate with the CVR (Fig. 2).

#### CVR in normal, Stage I and Stage II regions

The CVR was reduced in all six regions diagnosed as Stage II by Gas-PET (Fig. 3). However, CVR values were decreased in three of the 54 regions diagnosed as normal and in 3 of the 8 regions diagnosed Stage I (Fig. 3). Therefore, CVR was actually variable in the Stage I and normal groups.

#### Detection of Stage II cerebral hemodynamic failure using resting CBF and CVR

The three groups classified based on their OEF values were plotted according to CBF at rest and the CVR values obtained from the ACZ challenge H<sub>2</sub>O-PET study (Fig. 4). A CVR cut-off value of 0.19 (mean - 2SD) combined with a CBF cut-off value of 46.9 ml 100 g<sup>-1</sup> min<sup>-1</sup> (normal value) resulted in sensitivity and specificity for detecting misery perfusion of 86% (6/7) and 98% (61/62), respectively (OEF ≥ 53.3%). Therefore, the combination of the CVR and CBF appears to be a reliable index with which to accurately detect Stage II hemodynamic compromise in occlusive carotid diseases.

## DISCUSSION

The present study revealed a significant negative correlation between the CVR and the OEF and between the CVR and the CBV/CBF ratio in the MCA territories of patients with occlusive cerebrovascular disease. The CVR value of all regions with an elevated OEF was below 0.19. Additionally, the combination of CVR and CBF was highly sensitive and specific for detecting misery perfusion (OEF ≥ 53.3%). However, when the CBV/CBF ratio was elevated and OEF was normal (Stage I), the CVR was highly variable. These results support the notion that [<sup>123</sup>I]N-isopropyl-*p*-iodoamphetamine SPECT (<sup>123</sup>I-IMP SPECT) with ACZ challenge is clinically useful.<sup>9,10</sup>

Gibbs et al. applied the CBF/CBV ratio instead of vasodilative capacity as an indicator of autoregulatory vasodilation in brain tissue with decreased cerebral perfusion pressure.<sup>11</sup> The CBF/CBV ratio (or its inverse, the CBV/CBF ratio) is mathematically equivalent to the vascular mean transit time (MTT)<sup>5</sup> and could be more accurate than CBV alone as an index of cerebral perfusion pressure.<sup>12</sup> Although correlated with the CBV/CBF ratio, the CVR value was quite variable in the Stage I and normal groups. Some investigators have reported that CBV increases within the autoregulatory range,<sup>13-15</sup> while others have found minimal or no increases in CBV until autoregulatory capacity is exceeded.<sup>12,16</sup> CBV may be a complex physiological parameter, since it is composed of arterial, capillary and venous compartments.<sup>17</sup>

Based upon Powers' previous theory,<sup>4,5</sup> we originally surmised that CVR to ACZ would gradually decrease in the Stage I and would fall below zero in Stage II. However, the correlation between CVR and OEF in the present study was negative, suggesting that OEF may increase even at the stage when CVR in response to ACZ is still maintained. The correlation between CVR and OEF ( $r = -0.559$ ) was closer than that between CVR and CBV/CBF ( $r = -0.331$ ), suggesting that CVR can be an index reflecting both reduced perfusion pressure and increased OEF. In the modified model of hemodynamic and metabolic responses to a reduction in cerebral perfusion pressure after Powers et al.,<sup>4</sup> OEF can increase with reductions in CBF through autoregulatory ranges while CBV may not change.<sup>18,19</sup> The risk of ischemic stroke with increased OEF and increased CBV being higher than that with increased OEF and normal CBV suggests that OEF together with CBV can reflect hemodynamic compromise more precisely than OEF alone.<sup>19</sup> Recent studies have demonstrated the usefulness of the CVR to ACZ for predicting ischemic stroke.<sup>20</sup> Therefore, the CVR to ACZ challenge in H<sub>2</sub>O-PET combined with OEF in Gas-PET may be a better index with which to predict future stroke than OEF alone since more information about cerebral perfusion pressure is added.

In conclusion, the CVR to ACZ challenge could be influenced by both autoregulatory capacity and OEF.

Whether the combination of CVR with OEF may indeed add more predictive information about future ischemic stroke should be examined in a prospective study. The present results supported the notion that SPECT with ACZ challenge can be applied in clinical practice to detect patients with misery perfusion.

### ACKNOWLEDGMENTS

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## Significance of serum soluble thrombomodulin level in acute cerebral infarction

E. Nomura<sup>a</sup>, T. Kohriyama<sup>b</sup>, K. Kozuka<sup>c</sup>, H. Kajikawa<sup>d</sup>, S. Nakamura<sup>a</sup> and M. Matsumoto<sup>b</sup>

<sup>a</sup>Department of Neurology, Suiseikai Kajikawa Hospital, Hiroshima; <sup>b</sup>Department of Clinical Neuroscience and Therapeutics, Hiroshima University, Graduate School of Biomedical Sciences, Hiroshima; <sup>c</sup>Department of Neurology, Brain Attack Center, Oota Memorial Hospital, Fukuyama; and <sup>d</sup>Department of Neurosurgery, Suiseikai Kajikawa Hospital, Hiroshima, Japan

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thrombomodulin

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The purpose of the present study was to investigate sequential changes in serum soluble thrombomodulin (sTM) concentrations in patients with acute cerebral infarction (ACI), and to correlate sTM concentrations with the severity of ACI evaluated by Japan Stroke Scale. Eighty-three consecutive patients with ACI were enrolled, and blood examinations were carried out soon after admission and 1 month after. sTM concentrations at admission in patients with cardioembolic infarction ( $3.2 \pm 1.2$  ng/ml) were significantly lower than those of lacunar infarction ( $3.9 \pm 1.2$ ) ( $P < 0.05$ ). Serial examinations revealed that sTM concentrations increased significantly 1 month after admission ( $3.8 \pm 1.2$ ), compared with those at admission ( $3.6 \pm 1.2$ ) ( $P = 0.02$ ). Of three ACI subtypes, sTM concentrations during 1 month significantly increased in atherothrombotic infarction ( $P = 0.002$ ) or, not significantly, in cardioembolic infarction ( $P = 0.09$ ). The sTM concentrations at admission showed a significant inverse correlation with the severity of ACI ( $P = 0.04$ ). Although sTM concentrations serve as a useful marker for endothelial cell damage, they are decreased in patients with severe ACI, especially in atherothrombotic and cardioembolic infarctions. Lower sTM concentrations may play some important role in disease progression or in the recurrence following ACI, although the exact mechanism of this unique result should be clarified.

### Introduction

Acute cerebral infarction (ACI) is produced by thrombosis of the cerebral artery that leads to a reduced regional cerebral blood flow and neuronal ischemia, followed by neuronal cell death. Endothelial dysfunction plays an important role in thrombus formation, and cerebral thrombosis causes further endothelial damage, so that good markers for endothelial dysfunction/damage are at a high premium.

Thrombomodulin (TM), a receptor for thrombin, is a transmembrane glycoprotein located on the surface of vascular and lymphatic endothelium (Maruyama *et al.*, 1985; Esmon, 1989). When thrombin is bound to TM, fibrin formation, platelet activation (Esmon, 1989) and thrombin-mediated protein S cleavage (Mitchell *et al.*, 1986) are inhibited, and protein C is activated (Walker *et al.*, 1979). In addition to the membrane TM, this glycoprotein was reported to exist in soluble circulating forms in plasma. Interestingly,

these fragments together with membrane TM have been shown to exhibit an anticoagulant activity (Dittman, 1991). Soluble TM (sTM) levels increase in several pathologies when endothelial cells are damaged. Increased sTM levels have been reported amongst patients with peripheral and coronary artery disease (Blann and Seigneur, 1997). However, it has not been established whether acute ischemic stroke can affect sTM levels in the peripheral blood. The relation is not definite between sTM levels and severity or outcome of the acute ischemic stroke.

Recently, we reported in preliminary studies the several endothelial markers and adhesion molecules in acute ischemic stroke. In these studies, sTM concentrations during the acute stage did not increase, but became lower compared with the subacute (Kozuka *et al.*, 2002) or chronic stage (Nomura *et al.*, 2001).

The present study was performed with increased numbers of enrolled patients to exhibit the sequential change in sTM concentrations in each ACI subtype. Furthermore, we correlated these markers with the severity of stroke at admission, mortality within 1 month and the functional outcome 1 month after admission to elucidate the significance of sTM concentrations in ACI.

Correspondence: Dr Eiichi Nomura, Department of Neurology, Suiseikai Kajikawa Hospital, 8-20 Showa-machi, Naka-ku, Hiroshima City 730-0046, Japan (fax: (81) 82-249-5823; e-mail: nomura@mwc.biglobe.ne.jp).

## Subjects and methods

### Patients

Eighty-three consecutive patients with ACI admitted to the neurologic section in our hospital within 48 h from the onset were enrolled in the present study and followed up for 1 month. ACI was defined as a rapidly progressing focal neurologic deficit of vascular origin lasting more than 24 h, excluding hemorrhagic stroke by computed tomography performed at admission. Magnetic resonance imaging of the brain was immediately performed to detect the infarcted area. Patients were classified according to the Classification of Cerebrovascular Disease III (CVD III) of the National Institute of Neurological Disorders and Stroke (Committee Established by the Director of the NINDS, 1990), and consisted of three subtypes: atherothrombotic, cardioembolic and lacunar infarctions. Exclusion criteria were acute infection, malignancy and acute or chronic liver or kidney disease at admission. We selected controls without a past history of vascular events as references. Informed consent was obtained from all patients and control subjects.

### Clinical details

Blood examinations were carried out soon after admission and 1 month after admission. We tested the Japan Stroke Scale (JSS) (Gotoh *et al.*, 2001) at admission to evaluate the severity of ACI and the Functional Independence Measure (FIM) to evaluate the functional outcome of the patients 1 month after admission. JSS, which has been developed by the Japan Stroke Society, is the only weighted, parametric stroke impairment scale offering a quantitative measure of the severity of stroke. Reliability and responsiveness were proved to be excellent (Gotoh *et al.*, 2001). Patients were assigned as hypertension, if they are taking anti-hypertensive agents, or the systolic blood pressure is >160 mmHg or diastolic >95 mmHg, or both. Patients were diagnosed as diabetes mellitus, if they are either treated with oral hypoglycemic agents or insulin, or the serum fasting blood sugar level exceeds 140 mg/dl, or both. Hyperlipidemia was diagnosed, if they are taking antihyperlipidemic agents, or the serum cholesterol level exceeds 220 mg/dl, or both. All patients were followed up for 1 month except six patients who died as a direct consequence of ACI within 1 month after admission. We defined the patients scoring  $\geq 100$  on FIM 1 month after admission as good functional outcome ( $n = 45$ ) and  $< 100$  as poor functional outcome ( $n = 32$ ).

### Blood sampling and laboratory procedure

The serum sTM concentrations were determined at admission and 1 month after admission. Venous blood samples were drawn from the cubital vein of each subject, and were centrifuged for 10 min at 1200 g to obtain serum. Serum sTM concentration was measured by a sandwich enzyme immunoassay method using commercial monoclonal antibody-based enzyme-linked immunosorbent assay kits (TM Panacera; Fuji Chemical Industries Ltd, Tokyo, Japan). The monoclonal antibodies to human TM consisted of two types, recognizing different TM sites, and had been precoated onto a microplate. Standard samples and sera were pipetted into the wells, together with horseradish peroxidase (HRP)-conjugated anti-TM antibodies. After removal of unbound conjugated antibody, a substrate solution reactive to HRP was added to the wells. The absorbance of each well was measured with a spectrophotometer (Poseidon II; Aloka Co. Ltd, Tokyo, Japan). The concentration of sTM was obtained from the standard curve, plotting the optical density against the standard TM concentrations. The assay coefficients of variation between and within runs for TM were 4.2 and 3.0%, respectively.

### Statistical analysis

The data were analyzed with Stat View, version 5.0 (SAS Institute Inc., Cary, NC, USA), for Macintosh. Values were expressed as mean  $\pm$  standard deviation. The statistical significance of difference between two groups was tested with one-way ANOVA for continuous data or chi-square test for frequency data. Differences amongst three groups were tested with one-way ANOVA for continuous data, chi-square test for frequency data or Kruskal-Wallis test for ordinal data (FIM score). *Post hoc* test using Fisher's PLSD was used for continuous data. Sequential studies were analyzed by repeated measures ANOVA. Correlation between the sTM concentrations and the scores of JSS was calculated with the Spearman rank correlation coefficient. Values at  $P < 0.05$  were considered statistically significant.

## Results

Table 1 summarizes the clinical features of the patients and controls. Although the frequencies of hypertension and diabetes mellitus were significantly higher in patients compared with controls, the frequency of hyperlipidemia was not different between the two groups.

Table 2 summarizes the clinical features of the patients in each ACI subtype. The proportion of male and

**Table 1** Characteristics of patients and controls enrolled in the present study

	Controls	Patients	P-value
Number of subjects	66	83	
Age (mean $\pm$ SD)	65.8 $\pm$ 14.6	68.5 $\pm$ 10.2	0.2
Gender (male/female)	42/24	54/29	0.9
Death within 1 month		6	
Hypertension	20	45	0.004
Diabetes mellitus	7	24	0.006
Hyperlipidemia	12	22	0.2
JSS at admission (mean $\pm$ SD)		7.9 $\pm$ 7.0	
FIM 1 month after admission (median)		108	
sTM concentration (ng/ml)	3.5 $\pm$ 1.2		
At admission		3.6 $\pm$ 1.2	0.8
1 month after		3.8 $\pm$ 1.2	0.1

Differences between controls and patients were tested with one-way ANOVA for continuous data or the chi-square test for frequency data. JSS, Japan Stroke Scale; FIM, Functional Independence Measure.

female was not identical amongst three ACI subtypes, using chi-square test ( $P = 0.01$ ). Mean age and the frequencies of hypertension, diabetes mellitus and hyperlipidemia were not different amongst three groups. JSS score at admission (by one-way ANOVA) and FIM score 1 month after admission (by Kruskal-Wallis test) were not identical amongst three groups. *Post hoc* test revealed that the patients with lacunar infarction had significantly lower JSS scores at admission compared with those with atherothrombotic and cardioembolic infarction.

No significant difference in sTM concentrations was observed between controls and patients at admission or 1 month after admission (Table 1). sTM concentrations at admission in patients with cardioembolic infarction

(3.2  $\pm$  1.2 ng/ml) were significantly lower than those of lacunar infarction (3.9  $\pm$  1.2 ng/ml), using *post hoc* test (Table 2). Serial examinations revealed that sTM concentrations increased significantly 1 month after admission (3.8  $\pm$  1.2 ng/ml), compared with those at admission (3.6  $\pm$  1.2 ng/ml), using repeated measures ANOVA ( $F = 6.1$ ,  $P = 0.02$ ). Of three ischemic stroke subtypes, sTM concentrations during 1 month were significantly increased in atherothrombotic infarction ( $F = 11.5$ ,  $P = 0.002$ ) or, not significantly, in cardioembolic infarction ( $F = 3.4$ ,  $P = 0.09$ ) (Fig. 1).

There was no significant difference in sTM concentrations at admission between the deceased group (3.2  $\pm$  0.9 ng/ml) and survivor group (3.6  $\pm$  1.2 ng/ml), using one-way ANOVA ( $F = 0.6$ ,  $P = 0.5$ ).

The sTM concentrations at admission in patients with poor outcome (3.3  $\pm$  1.1 ng/ml) appeared to be lower than those with good outcome (3.8  $\pm$  1.3 ng/ml), using one-way ANOVA ( $F = 3.5$ ,  $P = 0.07$ ). The sTM concentrations in patients with poor outcome significantly increased 1 month after admission (3.9  $\pm$  1.3 ng/ml) and became almost equivalent to those of good outcome (3.8  $\pm$  1.1 ng/ml), using one-way ANOVA ( $F = 0.03$ ,  $P = 0.9$ ) (Fig. 2).

The sTM concentrations at admission showed a significant inverse correlation with the JSS scores at admission, calculated with a Spearman rank correlation coefficient ( $z = -2.1$ ,  $P = 0.04$ ) (Fig. 3).

## Discussion

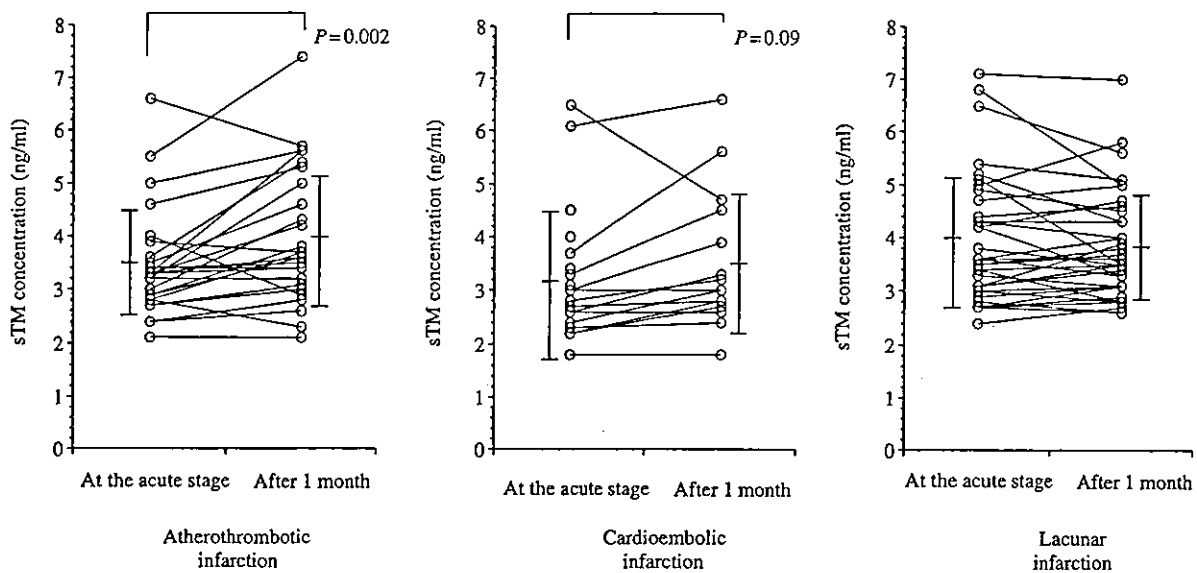
In the present study, we found that sTM concentrations were almost similar in patients with ACI as a whole compared with controls, and unexpectedly

**Table 2** Characteristics of patients in each cerebral infarction subtype

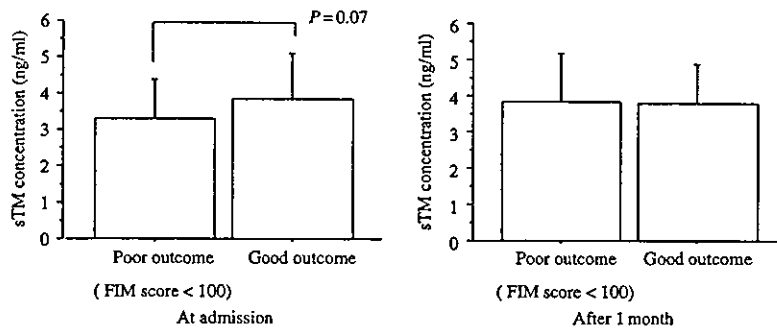
	Atherothrombotic infarction	Cardioembolic infarction	Lacunar infarction	P-value
Number of subjects	27	21	35	
Age (mean $\pm$ SD)	69.3 $\pm$ 10.2	68.4 $\pm$ 13.2	67.8 $\pm$ 8.0	0.8
Gender (male/female)	20/7	8/13	26/9	0.01
Death within 1 month	0	6	0	
Hypertension	16	12	17	0.7
Diabetes mellitus	7	4	13	0.3
Hyperlipidemia	7	4	11	0.6
JSS at admission (mean $\pm$ SD)	10.8 $\pm$ 5.6	13.3 $\pm$ 7.7	2.5 $\pm$ 2.0 <sup>a</sup>	<0.001
FIM 1 month after admission (median)	67	86	119	0.001
sTM concentration (ng/ml)				
At admission	3.4 $\pm$ 1.0	3.2 $\pm$ 1.2 <sup>b</sup>	3.9 $\pm$ 1.2	0.04
1 month after	4.0 $\pm$ 1.2	3.5 $\pm$ 1.3	3.8 $\pm$ 1.0	0.5

Differences amongst three groups were tested with one-way ANOVA for continuous data, chi-square test for frequency data or Kruskal-Wallis test for ordinal data (FIM score). *Post hoc* test was used for continuous data. JSS, Japan Stroke Scale; FIM, Functional Independence Measure.

<sup>a</sup>Significantly lower score compared with both atherothrombotic and cardioembolic infarction. <sup>b</sup>Significantly lower value compared with lacunar infarction.



**Figure 1** Sequential changes in sTM concentrations in each ACI subtype. sTM concentrations during 1 month were significantly increased in atherothrombotic infarction ( $F = 11.5, P = 0.002$ ) or, not significantly, in cardioembolic infarction ( $F = 3.4, P = 0.09$ ) using repeated measures ANOVA.

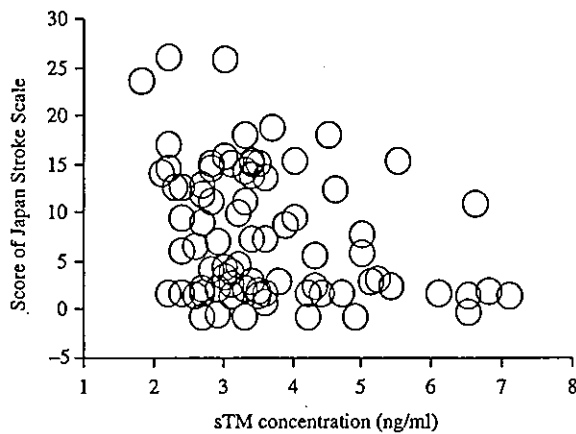


**Figure 2** Comparisons of sTM concentrations between poor and good functional outcome groups. sTM concentrations at admission in patients with poor functional outcome appeared lower than those of good functional outcome ( $n = 77, F = 3.5, P = 0.07$ ), and sTM concentrations between the two groups became similar 1 month after admission ( $n = 77, z = -0.03, P = 0.9$ ). Differences were tested using one-way ANOVA.

lowered at the acute stage in patients with serious condition.

The sTM levels increase in diseases caused by endothelial cell damage, including disseminated intravascular coagulation syndrome (Wada *et al.*, 1992), pulmonary embolism, respiratory distress syndrome, chronic renal insufficiency, acute hepatic insufficiency (Iwabuchi *et al.*, 1990; Takano *et al.*, 1990), diabetic angiopathy (Tanaka *et al.*, 1991), systemic lupus erythematosus (Takaya *et al.*, 1991), chronic myelogenous leukemia (Morishita *et al.*, 1992) and cancer (Lindahl *et al.*, 1993). Although increased sTM levels have also been reported amongst patients with

peripheral and coronary artery disease (Blann and Seigneur, 1997), it has not been established that sTM levels are elevated in patients with ACI. In the present and preliminary (Nomura *et al.*, 2001; Kozuka *et al.*, 2002) studies, the sTM levels in patients with ACI at admission did not increase, compared with controls. These results indicate that endothelial damage to the brain vessels may hardly cause elevated sTM levels. These may be supported by the finding that TM is absent in the brain parenchyma (Ishii *et al.*, 1986; Wang *et al.*, 1997) and is low (Ishii *et al.*, 1986) or distributed differently (Wang *et al.*, 1997) in the cerebral vessels.



**Figure 3** Relationship between Japan Stroke Scale (JSS) and sTM concentrations at admission. sTM concentrations at admission showed a significant inverse correlation with the JSS score at admission, calculated using the Spearman rank correlation coefficient ( $n = 83$ ,  $z = -2.1$ ,  $P = 0.04$ ).

We demonstrated lower sTM concentrations in patients with cardioembolic infarction than in patients with lacunar one at admission. Serial examination showed that sTM concentrations during 1 month increased significantly in atherothrombotic infarction and, not significantly, in cardioembolic infarction. Furthermore, sTM concentrations at admission showed a significant inverse association with the severity of the stroke measured by JSS, and the poor functional outcome group (scoring <100 on FIM) tended to have lower sTM concentrations at admission than the good functional outcome group (scoring  $\geq 100$  on FIM). Consequently, sTM levels seem possibly to be decreased during the acute stage, especially in serious patients with ACI.

We could postulate two possible mechanisms for the decrease in sTM concentrations of patients with ACI; one is consumption, the other, which is more probable, down-regulation. A marked elevation of plasma concentration of the thrombin-antithrombin III complex, reflecting the generation of thrombin, was reported in cardioembolic stroke (Takano *et al.*, 1992). Authors also revealed modest but significant decreases in the concentrations of antithrombin III and protein C, suggesting that these procoagulant inhibitors may have been consumed and consumption coagulopathy occurs in cardioembolic stroke (Takano *et al.*, 1991). The present study prompted us to consider that generated excess thrombin following ACI might cause significant decreases in sTM concentrations by consumption.

Thrombomodulin on cultured endothelial cells is up-regulated by cyclic adenosine monophosphate analogs (Hirokawa and Aoki, 1990), and down-regulated by tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (Conway and

Rosenberg, 1988), interleukin-1 (IL-1) (Nawroth *et al.*, 1986) or endotoxin (Moore *et al.*, 1987). It has been reported that TNF- $\alpha$  and IL-1 significantly increase in the rat ischemic cortex after temporary occlusion of the middle cerebral artery followed by reperfusion (Wang *et al.*, 1994). In addition, a recent study elucidated that an elevation in these proinflammatory cytokines in the peripheral blood was observed soon after ischemic stroke (Fassbender *et al.*, 1994; Ferrarese *et al.*, 1999; Vila *et al.*, 2000). Thus, sTM concentrations could conceivably be down regulated by these cytokines especially in acute ischemic stroke patients with large infarcts.

We thought that sTM levels could be decreased by similar mechanisms (down-regulation and/or consumption) in both atherothrombotic and cardioembolic infarctions. However, the number of enrolled patients with cardioembolic infarction was limited, and six patients with cardioembolic infarction (28.6%) died as a direct consequence of cerebral infarction before blood examination 1 month after. Further study of increased number of patients with cardioembolic infarction should be needed, and investigating the association between sTM and thrombin-antithrombin III complex, protein C, TNF- $\alpha$ , or IL-1 may be helpful to corroborate the hypothesis. Low sTM level plays an important role, accelerating the hemostatic unbalance toward a very potent prothrombotic state. Therefore, decreased sTM levels in ACI patients might cause the enlargement of the infarct area or the further development of vascular events. Conversely, we cannot deny another possibility that sTM concentration fluctuated physiologically and lower sTM concentration, triggering the ACI, was detected at the time of onset. However, the inverse association between sTM concentration and the stroke severity more strongly suggests that sTM concentration was affected by the incidence of cerebral infarction.

In conclusion, sTM levels did not increase in ACI, but rather decreased in atherothrombotic and cardioembolic infarctions. Lower sTM values were correlated with the severity at admission and seemed to associate with a poor functional outcome. The significance of these results should be investigated by further studies to clarify its mechanism.

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Short communication

## Cerebral ischemia in 5-lipoxygenase knockout mice

Kazuo Kitagawa<sup>a,\*</sup>, Masayasu Matsumoto<sup>b</sup>, Masatsugu Hori<sup>a</sup>

<sup>a</sup>Division of Strokeology, Department of Internal Medicine and Therapeutics (A8), Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan

<sup>b</sup>Department of Clinical Neuroscience and Therapeutics, Hiroshima University Graduate School of Biomedical Sciences, Hiroshima, Japan

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### Abstract

Cerebral ischemia induces 5-lipoxygenase translocation and leukotriene production in the brain. We tried to clarify the pathological significance of 5-lipoxygenase on cerebral ischemia using 5-lipoxygenase knockout mice. No significant difference was observed in the infarct size following permanent and transient ischemia for 60 min between both types of mice. The present study did not support the idea that leukotriene production is involved in infarct expansion in focal cerebral ischemia.

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*Theme:* Disorders of the nervous system

*Topic:* Ischemia

*Keywords:* Cerebral ischemia; 5-Lipoxygenase; Knockout mouse; Leukotriene

Arachidonic acid is released from membrane phospholipids by phospholipase A2 after cerebral ischemia and metabolized through three major enzymatic pathways [4,21,22]: cyclooxygenase (COX), lipoxygenase, and cytochrome P450 oxygenase. 5-Lipoxygenase together with five lipoxygenase activating protein (FLAP) [9] converts arachidonic acid to LTA<sub>4</sub>, which is hydrolyzed to LTB<sub>4</sub> or conjugated to glutathione to yield LTC<sub>4</sub>, LTD<sub>4</sub> and LTE<sub>4</sub>. These LTs induce a variety of responses including chemotaxis of leukocytes, smooth muscle contraction, and an increase in vascular permeability. In experimental cerebral ischemia, arachidonic acid is released from membrane phospholipid and converted enzymatically to products of the COX and lipoxygenase pathway [2,17]. We previously demonstrated the intracellular redistribution of 5-lipoxygenase from cytosolic to membranous fractions, which could make the enzymatic activity full [20]. Although the implication of 5-lipoxygenase on the brain edema after transient global ischemia has been investigated with lipoxygenase inhibitor [3,13,16], the pathological significance of 5-lipoxygenase in focal cerebral ischemia has not been fully

examined. Ciceri et al. [6] demonstrated recently that LT biosynthesis inhibitor MK-886 significantly reduced the cortical infarct size by 30% in permanent occlusion of middle cerebral artery in rats. In human autopsied brains, upregulation of 5-lipoxygenase has been shown in neurons, glial cells and mononuclear leukocytes during focal ischemic damage [23]. For a better understanding of the involvement of LT in focal cerebral ischemia, we used 5-lipoxygenase knockout mice and compared the infarct size after permanent and transient MCA occlusion between knockout and littermate mice.

5-Lipoxygenase knockout mice, originally produced by Chen et al. [5], were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). We first backcrossed them with the C57BL/6 mice (Charles River, Japan) more than five times to have genetic background of the homozygous and wild-type mice, and used them in the subsequent experiment. After mating heterozygotes, we selected the homozygous and wild-type mice by PCR amplification of genomic DNA extracted from the tail. All homozygous and wild-type mice used in the present study were mature males aged 12–16 weeks. Mice were given free access to food and water prior to surgery. The genotype was again confirmed after killing mice by immunoblotting of the lung homogenate using an antibody against 5-lipoxygenase (Cayman Chemical, Ann Arbor, USA). The experimental procedures involving labo-

\* Corresponding author. Tel.: +81-6-6879-3634; fax: +81-6-6878-6574.

E-mail address: [kitagawa@medone.med.osaka-u.ac.jp](mailto:kitagawa@medone.med.osaka-u.ac.jp) (K. Kitagawa).

ratory animals have been approved by the Institutional Animal Care and Use Committee of the Osaka University Medical School.

Twelve animals each of the wild-type and knockout mice were used for evaluation of infarct size. General anesthesia was induced with 2.0% halothane and maintained with 0.5% halothane by means of an open face mask. A polyacrylamide column with an inner diameter of 0.8 mm for measurement of cortical microperfusion by laser Doppler flowmetry (LDF, Advanced Laser Flowmetry, Model ALF-21, Advance) was attached to the intact skull, 3.5 mm lateral to the bregma with dental cement. A metal plate-type thermometer with a diameter of 3.0 mm was also attached to the skull over the parietal cortex to record skull temperature. A femoral artery was cannulated with a PE-10 polyethylene tube which was connected to a pressure

transducer attached to a calibrated polygraph, and body temperature was monitored with a rectal thermometer. Body and skull temperature was monitored and maintained at 36.0–37.5 °C and 35.0–36.5 °C, respectively, using a heat lamp.

The left MCA was exposed as described previously [12]. In brief, mice were placed in the recumbent position, and a vertical skin incision was made in the midpoint between the left orbit and the external auditory canal. The masseter muscles were dissected at the inferior edge of the zygoma, and the mandible was pulled downward to expose the skull base. Using a dental drill, a small burr hole was made in the skull over the left MCA. The dura was opened and the MCA was picked up. For permanent focal ischemia, the MCA was occluded using a micro-bipolar electrocoagulator just proximal to the point where

Table 1  
Physiologic variables and neurological scores after MCA occlusion

Permanent MCA occlusion			During ischemia				
Mouse strain		Preischemic	5 min	15 min	30 min	2 h	24 h
Wild type	Change in LDF (%)	100	22.8±7.1	23.5±5.0	22.0±4.9	–	–
	MABP (mm Hg)	75.8±5.3	72.0±4.3	70.5±3.5	73.0±5.7	–	–
	Body temperature (°C)	36.5±0.6	36.7±0.5	37.0±0.4	37.2±0.4	36.4±0.5	36.2±0.4
	Skull temperature (°C)	35.6±0.9	35.9±1.0	35.4±0.5	35.3±0.3	–	–
	Neurological Score	0	–	–	–	2.5±0.5	0.8±0.4
5-LO K/O	Change in LDF (%)	100	19.2±4.9	24.0±5.7	23.1±4.4	–	–
	MABP (mm Hg)	83.0±8.5	69.0±3.4	69.7±9.5	68.0±5.3	–	–
	Body temperature (°C)	36.2±0.6	36.8±0.2	37.0±0.3	37.2±0.3	36.4±0.2	36.8±0.4
	Skull temperature (°C)	35.2±0.4	36.2±0.9	35.6±0.5	35.9±0.7	–	–
	Neurological Score	0	–	–	–	2.8±0.4	1.3±0.8
Transient MCA occlusion			During ischemia				
Mouse strain		Preischemic	5 min	15 min	30 min	45 min	60 min
Wild type	Change in LDF (%)	100	21.3±7.3	25.2±5.7	24.2±5.4	24.2±7.4	22.4±5.3
	MABP (mm Hg)	76.2±8.9	74.0±7.6	69.7±7.6	68.7±6.1	71.0±3.3	69.3±6.2
	Body temperature (°C)	36.5±0.4	36.6±0.5	36.9±0.6	36.8±0.4	36.9±0.5	37.0±0.5
	Skull temperature (°C)	35.6±0.7	35.9±0.5	35.6±0.8	35.6±0.4	35.8±0.5	35.5±0.4
	Neurological Score	0	–	–	–	–	–
5-LO K/O	Change in LDF (%)	100	21.2±7.8	22.8±5.0	22.7±5.5	22.1±4.9	20.4±4.4
	MABP (mm Hg)	79.2±7.3	68.8±6.6	65.0±4.6	65.5±8.7	66.8±8.8	67.3±8.1
	Body temperature (°C)	36.0±0.6	36.2±0.8	36.7±0.5	36.9±0.8	36.9±0.8	36.7±0.6
	Skull temperature (°C)	35.3±0.4	35.8±0.4	35.5±0.4	35.9±0.6	35.9±0.9	35.7±0.4
	Neurological Score	0	–	–	–	–	–
Transient MCA occlusion			Recirculation				
Mouse strain			5 min	15 min	2 h	24 h	
Wild type	Change in LDF (%)		72.5±17.8	81.7±13.2	–	–	
	MABP (mm Hg)		68.2±2.4	69.8±5.5	–	–	
	Body temperature (°C)		36.7±0.5	37.3±0.4	36.1±0.2	36.0±0.7	
	Skull temperature (°C)		35.9±0.8	36.1±0.7	–	–	
	Neurological Score		–	–	2.5±0.8	0.8±0.4	
5-LO K/O	Change in LDF (%)		82.1±39.1	94.8±27.7	–	–	
	MABP (mm Hg)		69.7±5.8	69.7±4.4	–	–	
	Body temperature (°C)		36.7±0.4	36.8±0.5	36.4±0.4	36.3±0.8	
	Skull temperature (°C)		35.5±0.4	35.8±0.5	–	–	
	Neurological Score		–	–	2.7±0.8	0.8±0.4	

Values are shown as mean±S.D. based on six animals. Laser Doppler flowmetry (LDF), mean arterial blood pressure (MABP), 5-lipoxygenase-knockout mice (5-LO K/O).

the olfactory branch came off. For transient focal ischemia, the MCA was occluded temporarily with Zentyne miniclip for 60 min under halothane anesthesia and recirculated by clip removal. Cortical microperfusion was monitored for 30 min after permanent MCA occlusion and for 15 min after clip removal in transient MCA occlusion. After disconnection of halothane anesthesia, each mouse was allowed to recover for 2 h in a chamber where the ambient temperature was maintained at 35 °C to prevent hypothermia, and then each mouse was kept at room temperature. At 2 h and 1 day after MCA occlusion, mice were evaluated by a blinded observer for their neurological deficits. The neurological deficit score assignment of 0–4 was based on the methods described previously by Yang et al. [24]: 0, no neurological deficit; 1, failed to extend right forepaw while held by tail; 2, circled to the right; 3, fell to the right; 4, unable to walk spontaneously.

Seven days after MCA occlusion, mice were killed with an overdose of pentobarbital. The whole brains were removed, fixed by immersion into an alcohol–5% acetic acid solution for 5 h at 4 °C, dehydrated, and embedded in paraffin. Tissue sections (5 µm) were obtained every 1 mm, beginning at the frontal pole, and were examined after conventional staining with hematoxylin and eosin. The volume of ischemic infarction was measured using an MCID image analysis system (Imaging Research, St. Catharines, Ontario, Canada). The infarction areas were calculated by tracing the areas on a computer screen. The volume (mm<sup>3</sup>) was determined by integrating the appropriate area and the section thickness.

All values reported here are expressed as mean±S.D. Statistical significance of differences among groups was tested by unpaired *t*-test.

Cortical microperfusion in the ischemic center after MCA occlusion was 15–30% of the baseline value in both permanent and transient occlusion. In transient MCA

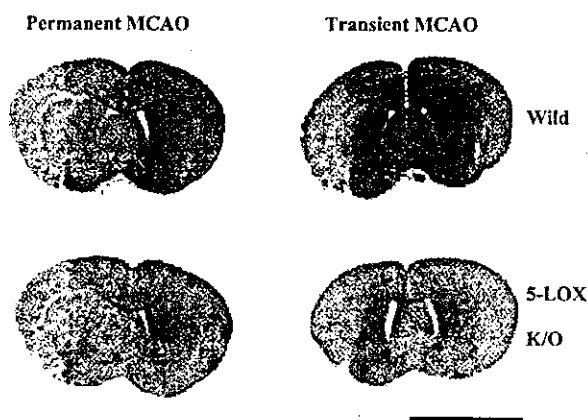


Fig. 1. Hematoxylin and eosin (HE) staining in the whole brain of wild-type (Wild) and 5-lipoxygenase knockout mice (5-LOX K/O). Permanent middle cerebral artery occlusion (MCAO) (left column). Transient middle cerebral artery occlusion. The extent of infarct area was similar in both types of mice (right column). Bar=0.5 mm.

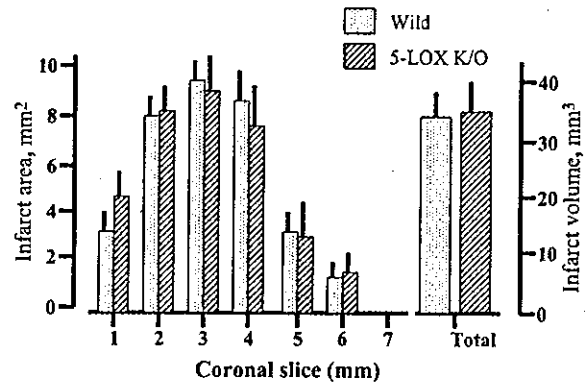


Fig. 2. Infarct area and size after permanent MCA occlusion. Infarct area in seven coronal sections from rostral to caudal (1–7 mm) and infarct volume shown for wild-type (stippled column) and 5-lipoxygenase knockout mice (hatched column). Infarct area and size was almost the same between the two types of mice.

occlusion, cortical microperfusion recovered to 80–90% of the baseline value 15 min after clip removal. The values were similar between knockout and wild-type mice (Table 1). Other physiologic parameters, including blood pressure and body and skull temperatures, were maintained similarly between both types of mice (Table 1). Infarct areas after both permanent and transient MCA occlusion were almost the same for both types of mice on the coronal slices 3 mm from the frontal pole (Fig. 1). The total infarct volume also was similar between both types of mice in permanent (wild type, 33.0±3.3 mm<sup>3</sup>, *n*=6; knockout mice, 33.6±4.4 mm<sup>3</sup>, *n*=6; *P*=0.45) (Fig. 2) and transient ischemia (wild type, 13.8±6.3 mm<sup>3</sup>, *n*=6; knockout mice, 14.4±5.6 mm<sup>3</sup>, *n*=6; *P*=0.51) (Fig. 3). The neurological deficit score was also similar between both types of mice in permanent and transient ischemia (Table 1).

After cerebral ischemia, arachidonic acid is released from membrane phospholipid and enzymatically converted to prostaglandin and thromboxane through cyclooxygenase, and to hydroperoxyeicosatetraenoic acid (HPETE) through 5-, 12-, or 15-lipoxygenase. 5-Lipoxygenase is a key enzyme for production of LTs which constitute a class of potent biological mediators of inflammation and anaphylaxis. 5-Lipoxygenase knockout mice resist the lethal effects of shock by platelet-activating factor, and show reduced reaction to ear inflammation induced by arachidonic acid [5,10]. It was recently demonstrated that those mutant mice also exhibited a resistance to splanchnic artery occlusion shock [8]. Because the phenotype of 5-lipoxygenase knockout mice is useful to identify the role for leukotrienes in the pathophysiology under injurious insult, we used this mouse strain to investigate the role of 5-lipoxygenase and leukotriene in ischemic brain damage. In this study, we used both permanent and transient focal ischemia to mimic human stroke. The role of LT on ischemic brain damage has been examined with lipoxygenase inhibitor mostly in transient global ischemia model.

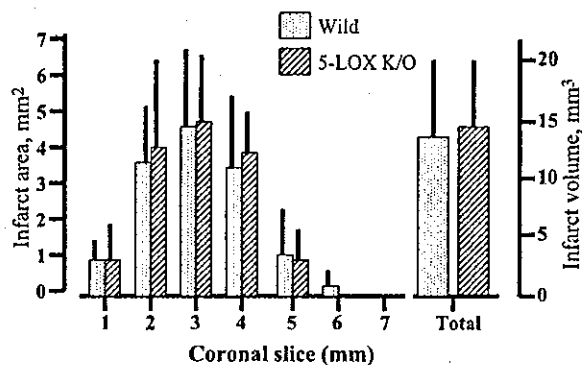


Fig. 3. Infarct area and size after transient MCA occlusion. Infarct area and infarct volume for wild-type (stippled column) and 5-lipoxygenase knockout mice (hatched column).

Several studies supported the involvement of LT on brain edema after ischemia [3,13,16]; however, the effect of lipoxygenase inhibitor on ischemic neuronal damage is controversial [1,7,18,21]. Furthermore, there are few studies examining the role of LT in focal cerebral ischemia. Ciceri et al. [6] demonstrated that LT biosynthesis inhibitor MK-886 decreased LT level and reduced the cortical infarct size by 30%, 24 h after permanent MCA occlusion. However, in our study, no significant difference was observed in the infarct size between wild type and 5-lipoxygenase knockout mice. Although we maintained blood pressure and body temperature and monitored change in cortical microperfusion after vessel occlusion, no information for such physiological parameters was shown in Ciceri et al.'s [6] study, making it difficult to compare our results with their study. Furthermore, infarction certainly expands during a few days after MCA occlusion in a rat [14,15]; therefore, evaluation of infarct size 24 h after occlusion in Ciceri et al.'s [6] study may look over infarct expansion. Although no protection against cerebral ischemia was found in 5-lipoxygenase knockout mice in this study, potential beneficial effect by suppression of LT production would be set off by increased substrate availability for cyclooxygenase (COX) pathway. Among two COX isoforms, COX2 has been shown a key molecule for ischemic brain damage. Both inhibition of COX2 activity with COX2-specific inhibitor and deficiency of COX2 in knockout mice showed protection against ischemic brain damage [11,19]. Furthermore, 12-HPETE and 15-HPETE and their metabolites, lipoxins, produced by 12- and 15-lipoxygenase may increase and contribute to infarct expansion in 5-lipoxygenase knockout mice. However, our result does not exclude the possibility that inhibitor of 5-lipoxygenase or leukotriene antagonist may have potential as a therapeutic target against stroke. Inhibition of COX can increase substrate availability for 5-lipoxygenase and LT production which aggravate vascular permeability and brain edema. Combination of COX2 inhibitor with lipoxygenase inhibitor or

LT antagonist in experimental stroke model may be worth consideration.

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