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Thrombolysis With 0.6 mg/kg Intravenous Alteplase for Acute Ischemic Stroke in Routine Clinical Practice

The Japan post-Marketing Alteplase Registration Study (J-MARS)

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Background and Purpose—In Japan, alteplase at 0.6 mg/kg was approved in October 2005 for use within 3 hours of stroke onset by the Ministry of Health, Labor and Welfare (MHLW). The aim of the Japan post-Marketing Alteplase Registration Study (J-MARS), which was requested by MHLW at the time of approval, was to assess the safety and efficacy of 0.6 mg/kg alteplase in routine clinical practice for the Japanese.

Methods—A total of 7492 patients from 942 centers were enrolled in the J-MARS, an open-label, nonrandomized, observational study, from October 2005 to October 2007. Primary outcome measures were symptomatic intracranial hemorrhage (a deterioration in NIHSS score ≥4 from baseline) and favorable outcome (modified Rankin Scale score, 0–1) at 3 months after stroke onset.

Results—The proportion of patients with symptomatic intracranial hemorrhage in 7492 patients (safety analysis) was 3.5% (95% confidence interval [CI], 3.1%–3.9%) within 36 hours and 4.4% (95% CI, 3.9%–4.9%) at 3 months. The overall mortality rate was 13.1% (95% CI, 12.4%–13.9%) and the proportion of patients with fatal symptomatic intracranial hemorrhage was 0.9% (95% CI, 0.7%–1.2%). The outcomes at 3 months were available for 4944 patients and the proportion of favorable outcome (efficacy analysis) was 33.1% (95% CI, 31.8%–34.4%). The subgroup analysis in patients between 18 and 80 years with a baseline NIHSS score <25 demonstrated that favorable outcome at 3 months was 39.0% (95% CI, 37.4%–40.6%).

Conclusions—These data suggest that 0.6 mg/kg intravenous alteplase within 3 hours of stroke onset could be safe and effective in routine clinical practice for the Japanese. (***Stroke.* 2010;41:1984-1989.**)

Key Words: acute ischemic stroke ■ alteplase ■ postmarketing registration ■ thrombolysis ■ tissue plasminogen activator

Since the recombinant tissue plasminogen activator stroke study organized by the National Institute of Neurological Disorders and Stroke (NINDS)[1] demonstrated that intravenous alteplase treatment within 3 hours of stroke onset improved functional outcome in 1995, this treatment has been an approved medical therapy for patients with acute ischemic stroke and is recommended as the first-line treatment by most national and international guidelines.[2,3] Intravenous alteplase treatment of ischemic stroke within the 3-hour time window has been shown to be safe and effective in previous randomized controlled trials.[4–8] However, the safety and efficacy of thrombolysis with alteplase in routine clinical practice should be investigated in each country.

Alteplase was licensed for the treatment of acute ischemic stroke in the United States in 1996 and in the European Union in 2002 for selected patients treated within the 3-hour time window. In Japan, a prospective, single-arm, open-label study called the Japan Alteplase Clinical Trial (J-ACT)[9] was conducted from April 2002 to September 2003. Although the internationally recommended dosage of intravenous alteplase was adjusted to 0.9 mg/kg, the challenging dose of 0.6 mg/kg was selected in J-ACT based on previous recombinant tissue plasminogen activator studies for Japanese patients. Randomized controlled trials of duteplase, a recombinant tissue plasminogen activator similar to alteplase, have been conducted for acute stroke patients within 6 hours of onset in

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Japan.[10–12] After a pilot study,[10] 20 million international units (MIU) of duteplase proved to be superior to placebo based on the angiographic recanalization rate.[11] In a randomized, double-blind, dose-comparison study, partial recanalization and complete recanalization in 18 of 54 (33.3%) patients administered 20 MIU and in 25 of 59 (42.4%) patients administered 30 MIU, respectively, were not found to be statistically different.[12] However, massive brain hematoma/hemorrhagic transformation occurred in 2 of 56 (3.6%) patients administered 20 MIU and 9 of 65 (13.8%) patients administered 30 MIU.[12] Therefore, it was considered that the optimal dose of alteplase for J-ACT was 0.6 mg/kg, which was equivalent to 20 MIU per person or 0.33 MIU/kg at a mean body weight of 60 kg. The underlying rationale has been published on the Stroke web site (http://stroke.ahajournals.org/cgi/content/full/37/7/1810).[9] In J-ACT, 103 patients were treated with 0.6 mg/kg intravenous alteplase, and the proportion of modified Rankin Scale (mRS) score of 0 to 1 at 3 months was 36.9% (38/103; 90% confidence interval [CI], 29.1%–44.7%), and the incidence of symptomatic intracranial hemorrhage (sICH) within 36 hours was 5.8% (6/103; 90% CI, 2.0% to 9.6%).[9] Consequently, alteplase at 0.6 mg/kg was approved and a license was granted in October 2005 by the Ministry of Health, Labor and Welfare (MHLW), Japan. At the time of approval, the MHLW required the sponsors (Mitsubishi Tanabe Pharma Corporation and Kyowa Hakko Kirin Co, Ltd) to perform a large-scale postmarketing registry study to assess the safety profile of 0.6 mg/kg intravenous alteplase and a clinical study for documentation of the dosage efficacy (Japan Alteplase Clinical Trial II [J-ACT II]).[13] The sponsors asked the centers practicing thrombolysis with alteplase for participation in the postmarketing registry. The results of both studies will contribute to a standard for the reassessment of the benefit-to-risk profile of intravenous alteplase treatment.

The aim of Japan post-Marketing Alteplase Registration Study (J-MARS) was to investigate whether thrombolysis with 0.6 mg/kg intravenous alteplase could be safe and effective in routine clinical practice for the Japanese. Here, we compared the results of J-MARS with those of the Safe Implementation of Thrombolysis in Stroke-Monitoring Study (SITS-MOST) performed as a postmarketing study in the European Union.[14]

Patients and Methods

J-MARS was an open-label, multicenter, nonrandomized, observational study including clinical centers practicing thrombolysis for acute stroke in Japan. Participation in this study was possible for any medical centers that committed to register all patients treated with alteplase for 2 years after its approval and to collaborate in the elucidation of causes of any treatment complications. Joining this registry was not compulsory. The primary outcome measures in the protocol were sICH within 36 hours and at 3 months and favorable outcome (mRS score, 0–1) at 3 months after stroke onset. The MHLW approved the protocol of this study and the sponsors instructed the investigators to perform the study according to Good Postmarketing Study Practice, which is the authorized standard for a postmarketing registration study. The ethics approval was obtained from institutional ethics committee when required. Thrombolysis with 0.6 mg/kg intravenous alteplase was applied for the patients in accordance with the existing labeling and guidelines for intravenous alteplase treatment in Japan.[15,16] Informed consent was obtained from the patient (or a relative if the patient could not understand the treatment). Recruitment of patients in J-MARS started in October 2005 and ended in October 2007.

Baseline and demographic characteristics, stroke severity, time intervals, risk factors, and medication history were collected. NIHSS score at 24 hours and mRS score at 3 months were requested as the outcome measures. The proportion of each mRS score at 3 months was also calculated. Any adverse events for patients in this study were reported via their case report forms (CRF) to the sponsors, who reported serious drug-related adverse reactions to MHLW.

All patients who were enrolled in this study underwent CT or MRI before and within 36 hours after treatment as a general rule. Further follow-up brain scans after that were optional; however, patients who presented neurological deterioration underwent additional scan. These scans were not reviewed centrally. sICH was defined as any intracranial hemorrhage with a neurological deterioration of NIHSS score ≥4 points from baseline, or from the lowest NIHSS score after baseline to 24 hours, or the intracranial hemorrhage leading to death. In addition, number of patients with sICH was stratified according to number of enrolled patients per center. Functional independence (mRS score, 0–1) was assessed at 3 months after stroke onset by face-to-face or telephone interview with the patient or the patient's caregiver, or by letter reply form. Intracranial hemorrhage rates were calculated from any CT or MRI within 36 hours after alteplase treatment, and also from any additional scans.

Statistical Analysis

The proportion and 95% CI of patients with sICH, favorable outcome, and mortality rate were calculated. We used the statistical approach to calculate the upper and lower limits of the CI. Bar charts of proportions of patients were made to compare with the corresponding proportions of the NINDS study,[1] the J-ACT,[9] the SITS-MOST,[14] the Standard Treatment with Alteplase to Reverse Stroke study (STARS),[17] and the Canadian Alteplase for Stroke Effectiveness Study (CASES).[18] All analyses were performed with SAS version 9.1.3.

Results

According to the logistics research, 8313 patients with acute ischemic stroke at 1100 centers were treated with intravenous alteplase from October 2005 to October 2007 all over Japan, and a total of 7692 patients from 959 centers were registered in J-MARS. However, 200 patients from 83 centers (2.6%; 200/7692) whose CRF were not collected because of nonfulfillment by the investigators were excluded. Finally, 7492 patients (90%; 7492/8313) with CRF from 942 centers (86%; 942/1100) were enrolled in the safety analysis (Figure 1). The proportion of patients with sICH, prestroke independence (mRS score, 0–1), and functional outcomes at 3 months were obtained from the CRF. The overall mortality rate was estimated from the fatal records in the CRF. The median participated time in the registry for these 942 centers was 17.9 months. Table 1 shows baseline characteristics in 7492 patients, including risk factors, presence of concomitant disease, degree of neurological severity, and blood pressure in J-MARS in comparison with SITS-MOST. Table 1 also shows stroke subtypes of the subjects and median time from stroke onset to alteplase treatment in both studies.

Table 2 demonstrates the rates of adverse events, drug-related adverse reactions, intracranial hemorrhages confirmed by brain scans, and overall mortality in 7492 patients. The proportion of patients with sICH was 3.5% (259/7492; 95% CI, 3.1%–3.9%) within 36 hours and 4.4% (329/7492; 3.9%–4.9%) at 3 months. The overall mortality rate within 3 months was 13.1% (985/7492; 12.4%–13.9%) and the proportion of patients with fatal sICH was 0.9% (70/7492; 0.7%–1.2%).

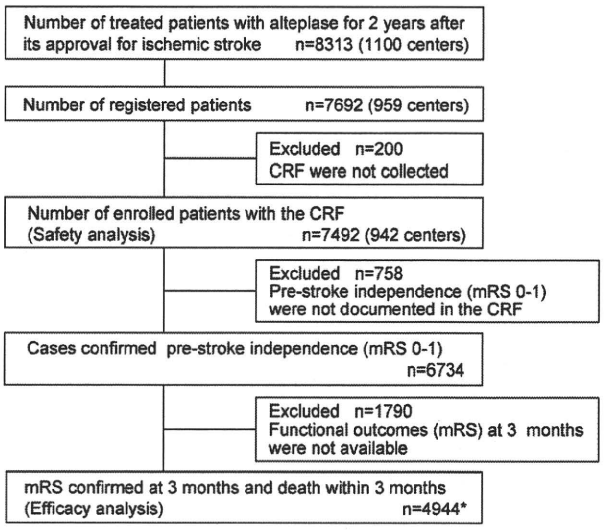

Figure 1. Flow chart showing the disposition of patients. *Of 4944 patients, modified Rankin Scale (mRS) scores at 3 months for 4060 patients were documented in their case report forms, and those of 884 patients were confirmed by attending physicians and records at hospital discharge.

Regarding neurological outcome in J-MARS, the median NIHSS score was 15 (interquartile range, 9–20) at baseline and 10 (interquartile range, 4–18) at 24 hours from starting therapy. Of the total 7492 patients, 758 patients had no documentation for prestroke independence (mRS score, 0–1) in the CRF (Figure 1). In these 758 patients, prestroke disability states of 741 patients were reported as mRS score 2 to 5, and those of remaining 17 patients were not mentioned. They were included in the safety analysis but not in the efficacy analysis, because favorable outcome was defined as mRS score 0 to 1 in this study. Number of patients who confirmed prestroke independence (mRS score, 0–1) was 6734. Follow-up data at 3 months were available for 4944 of 6734 patients whose prestroke independence was confirmed (Figure 1); 1790 of 6734 patients were excluded from the efficacy analysis because their mRS scores at 3 months were not available. Functional outcomes at 3 months (90±14 days) were obtained in 4060 of 4944 patients (including virtually all deceased cases within 3 months). For the other 884 patients who were surviving at 3 months, their functional outcomes were unavailable in the CRF, but their mRS score were confirmed by attending physicians and records at hospital discharge. The proportion of favorable outcome at 3 months in J-MARS was 33.1% (1637/4944; 31.8%–34.4%). The functional outcome estimated by mRS score at 3 months was compared with data from relevant published studies in Figure 2.

Table 1. Baseline Characteristics of Patients Analyzed in J-MARS and SITS-MOST

	J-MARS (n=7492)	SITS-MOST (n=6483)
Age, y	72 (65–79)	68 (59–75)
Gender, female	2836 (37.9%)	2581 (39.8%)
Prestroke independence, mRS score 0–1	6734 (89.9%)	5899/6337 (93.1%)
Concomitant disease		
Hypertension	3852 (51.4%)	3710/6318 (58.7%)
Diabetes mellitus	1272 (17.0%)	1020/6374 (16.0%)
Atrial fibrillation	3331 (44.5%)	1507/6306 (23.9%)
Heart failure	679 (9.1%)	467/6339 (7.5%)
Previous stroke	1373 (18.3%)	643/6395 (10.1%)
NIHSS score	15 (9–20)	12 (8–17)
Systolic blood pressure, mm Hg	150 (136–164)	150 (137–166)
Diastolic blood pressure, mm Hg	81 (71–90)	81 (74–90)
Stroke subtype		
Cardioembolic	4509 (60.2%)	2270 (35%)
Atherothrombotic	1838 (24.5%)	2279 (35.2%)*
Lacunar	316 (4.2%)	535 (8.3%)
Other/not differentiated	811 (10.8%)	1171 (18.1%)
Unknown	18 (0.2%)	228 (3.5%)
Stroke onset to treatment time, min	133 (110–160)	140 (115–165)

Data are median (interquartile range) or n (%).

*Large vessel disease with or other than substantial carotid stenosis.

J-MARS indicates Japan post-Marketing Alteplase Registration Study; mRS, modified Rankin scale; SITS-MOST, Safe Implementation of Thrombolysis in Stroke-Monitoring Study.

Table 2. N (%) of Patients With All Adverse Events, Drug-Related Adverse Reactions, Intracranial Hemorrhage, and Overall Mortality

Total N of Patients	7492
Adverse events	2412 (32.2%)
Drug-related adverse reactions	1627 (21.7%)
Intracranial hemorrhages	1217 (16.2%)
sICH	
Within 36 hr	259 (3.5%)
At 3 mo	329 (4.4%)
Fatal sICH	70 (0.9%)
Overall mortality	985 (13.1%)

sICH, symptomatic intracranial hemorrhages.

The median NIHSS score at baseline was 15 for J-MARS (n=3576) and 12 for SITS-MOST (Figure 2). The proportion of patients with NIHSS score ≥25 at baseline was 9.4% (463/4944) in J-MARS. The proportion of patients with alteplase treatment initiated later than 3 hours after symptom onset was 1.8% (91/4944).

In SITS-MOST, the subjects were restricted to those between ages 18 and 80 years with an NIHSS score <25.[14] The subgroup analysis with selected conditions such as those of SITS-MOST showed that the proportion of favorable outcome at 3 months was 39.0% (37.4%–40.6%) in J-MARS (n=3576) in comparison with 38.9% (37.7%–40.1%) in SITS-MOST (Figure 3).

We stratified number of patients with sICH according to number of enrolled patients per center (Figure 4). The percentage of sICH for centers with a small enrolled number (≤4) was 6.0% (4.7%–7.7%), and those for centers with a relatively larger enrolled number (20–29 and ≥30) were 3.2% (2.3%–4.4%) and 3.2% (2.4%–4.2%), respectively.

Discussion

The results from J-MARS suggested that 0.6 mg/kg intravenous alteplase could be an effective treatment with satisfac-

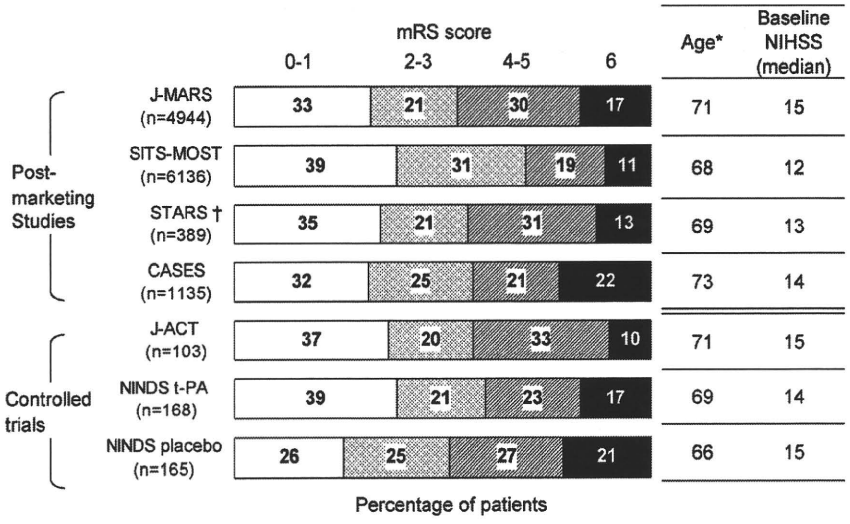

Figure 2. modified Rankin Scale (mRS) score at 3 months in J-MARS, other postmarketing studies, and controlled trials. *Data of J-MARS, SITS-MOST, and CASES are medians. The other data are means. †The mRS score at 30 days in STARS.

tory safety profile when used in a 3-hour time window in routine clinical practice for the Japanese. For the first 2 years after the approval of intravenous alteplase treatment in Japan, most patients who received this treatment were registered in J-MARS. The main aim of J-MARS was to confirm whether the levels of safety recognized in published clinical studies could be reproduced in routine clinical practice, especially with regard to sICH.

In J-MARS, the proportion of patients with sICH was 4.4% (3.9%–4.9%) at 3 months. The definitions of sICH have been slightly different among published studies.[1,9,14,17,18] These differences could restrict any direct comparison of the results from those studies. In SITS-MOST, the proportion of patients with sICH was 7.3% (6.7%–7.9%) according to the National Institute of Neurological Disorders and Stroke and Cochrane review definition[19,20] (defined as any hemorrhage plus any neurological deterioration [NIHSS score ≥1] or that leads to death within 7 days) and 4.6% (4.1%–5.1%) according to the European Cooperative Acute Stroke Study (ECASS) definition[21] (defined as any hemorrhage plus a neurological deterioration of NIHSS score ≥4 points from baseline, or from the lowest NIHSS score after baseline to 7 days or leading to death).[14] Our results showed that the proportions of patients who had sICH in J-MARS and SITS-MOST were comparable when the ECASS definition was applied to those in SITS-MOST (4.4% vs 4.6%).

In J-MARS, we stratified number of patients with sICH according to number of enrolled patients from each participating center to investigate the correlation between the experience and the safety with stroke thrombolysis. The number of enrolled patients per center in J-MARS was small compared to that of SITS-MOST, but the percentage of sICH in centers with a relatively large number (≥20 cases) of enrollment was lower than that in centers with relatively small number (≤19 cases) of enrollment (Figure 4). This finding suggested that the experience of stroke thrombolysis was one important factor for safe clinical practice.

The proportion of favorable outcome at 3 months in J-MARS remained at 33.1%, which is nearly the same rate as that seen in CASES, in which favorable outcome was 32%.[18] The modest data collection rate of functional outcome evaluations at 3 months (4944/7492) seems to be an inevitable limitation of this observational study and could be a possible source of detection and exclusion biases. Although mRS scores for surviving patients at 3 months were not always reported in the CRF, virtually all fatal cases within 3 months were identified in the fatal records in the CRF. Accordingly, the proportion of mRS score 6 at 3 months (17% in Figure 2)

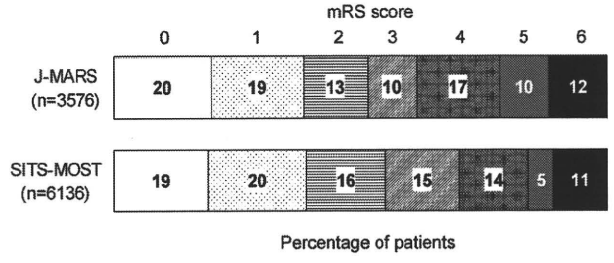

Figure 3. modified Rankin Scale (mRS) score at 3 months in subgroup restricted to patients between ages 18 and 80 years with NIHSS score <25 in J-MARS in comparison with SITS-MOST. The actual numbers of patients in each category for J-MARS (n=3576): mRS score 0, 704; mRS score 1, 690; mRS score 2, 466; mRS score 3, 344; mRS score 4, 598; mRS score 5, 363; mRS score 6, 411. Median ages: J-MARS, 69 years; SITS-MOST, 68 years. Median of baseline NIHSS score: J-MARS, 13; SITS-MOST, 12.

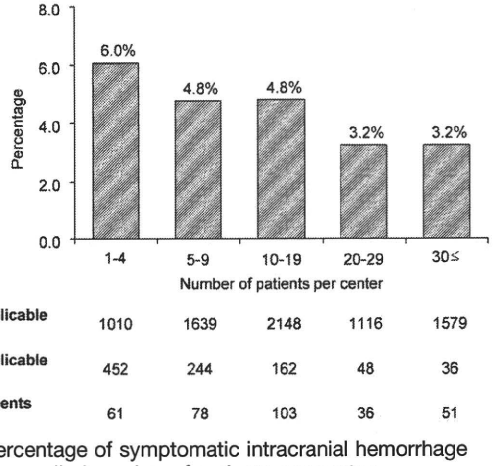

Figure 4. Percentage of symptomatic intracranial hemorrhage according to enrolled number of patients per center.

was seemingly higher than the overall mortality rate (13.1% in Table 2). Certainly, mortality at 3 months in J-MARS was higher than that in J-ACT (10% in Figure 2).[9] The median NIHSS score at baseline was 15 in J-MARS, which is the same value as in J-ACT (Figure 2). In J-ACT, patients with a comatose state at baseline were excluded, and the highest NIHSS score at baseline was actually 30. However, a considerable number of patients with the severe baseline condition of NIHSS score >30 or with a comatose state were included in J-MARS, and their outcomes were almost always unfavorable.

In SITS-MOST, the proportion of favorable outcome at 3 months was 39%.[14] Concerning the relatively higher favorable outcome in SITS-MOST, it could be a contributing factor that study recruitment was restricted to patients between ages 18 and 80 years with NIHSS score <25. In SITS-MOST, the median age was 68 years (vs 72 years in J-MARS), and the median NIHSS score was 12 (vs 15 in J-MARS).[14] Thus, clinical severity could be less severe in SITS-MOST. Consequently, we tried the subgroup analysis of J-MARS in patients between ages 18 and 80 years with an NIHSS score <25, which demonstrated that the favorable outcome at 3 months was 39%, which is much the same as that in SITS-MOST (39%; Figure 3).

Recently, the Stroke Acute Management with Urgent Risk-factor Assessment and Improvement (SAMURAI) study was conducted in 10 Japanese stroke centers with much experience in alteplase treatment from October 2005 to July 2008.[22] Six hundred patients treated with 0.6 mg/kg intravenous alteplase were enrolled in SAMURAI study and they were partially overlapped with those in J-MARS. In SAMURAI study, the proportion of favorable outcome at 3 months was 33.2% (29.5%–37.0%) of the total 600 patients and 37.2% (33.2%–41.4%) when 65 patients with a prestroke mRS score 2 to 5 were excluded from the analysis. Analysis of 399 patients with a prestroke mRS score 0 to 1 who met the criteria of SITS-MOST showed that the proportion of favorable outcome at 3 months was 40.6% (35.9%–45.5%). Although SAMURAI study group was composed of stroke centers with much experience in alteplase treatment, the proportion of favorable outcome in SAMURAI study was not so superior to that of the present study. The results of J-MARS, the national postmarketing study in Japan, could be positively ranked with those of SITS-MOST.

Conclusion

In conclusion, the result of J-MARS demonstrated that 0.6 mg/kg intravenous alteplase achieved low rates of sICH and sufficient favorable outcome in clinical practice in Japan. In addition, the results from J-ACT II showed that early recanalization of an occluded middle cerebral artery was generated by 0.6 mg/kg intravenous alteplase and directly associated with favorable clinical outcome.[13] The results of these Japanese studies suggest that thrombolysis with 0.6 mg/kg intravenous alteplase could be comparable to those with 0.9 mg/kg alteplase used in North America and the European Union. Hereafter, the safety and efficacy of thrombolysis with 0.6 mg/kg intravenous alteplase could contribute not only to routine clinical practice but also to occasional combined approach with thrombolysis and endovascular devises for patients with acute ischemic stroke.

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Carotid Duplex Ultrasonography Can Predict Outcome of Intravenous Alteplase Therapy for Hyperacute Stroke

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We evaluated whether carotid duplex ultrasonography (US) can help predict the safety and efficacy of treating hyperacute stroke with intravenous (IV) tissue plasminogen activator (alteplase) therapy. Consecutive patients with stroke were assigned to the carotid artery occlusion (CO) group or the other (non-CO) group according to US findings before or immediately after receiving IV alteplase. Effectiveness and safety outcomes included early neurologic improvement, defined as a reduction in a National Institutes of Health Stroke Scale (NIHSS) score of ≥4 points within the initial 24 hours after stroke onset; completely independent routine activity, defined as a modified Rankin Scale score of ≤1 at day 90 after stroke onset; symptomatic intracranial hemorrhage (ICH) occurring within 36 hours after stroke onset; and any ICH. We enrolled 127 patients (27 in the CO group and 100 in the non-CO group) with a median baseline NIHSS score of 13 (range, 4-30). The CO group had a higher baseline NIHSS score (median, 18 vs 12; $P = .005$). After multivariate adjustment, the CO group was inversely associated with early improvement (odds ratio [OR] = 0.26; 95% confidence interval [CI] = 0.09-0.72) and independence at day 90 (OR = 0.23; 95% CI = 0.05-0.73) and positively associated with any ICH (OR = 3.11; 95% CI = 1.23-8.48). Our findings indicate that CO identified by US in the emergency clinical setting is an independent predictor of unfavorable outcome and ICH following IV alteplase therapy. **Key Words:** Alteplase—internal carotid artery occlusion—intracranial hemorrhage—ultrasonography—outcome.

Occlusion of the internal carotid artery (ICA) often provokes severe hypoperfusion of cerebral blood flow in the affected territory. Patients who sustain acute ICA occlusion tend to have poor clinical outcomes.[1] Mortality is high in patients with malignant middle cerebral artery (MCA) infarction, resulting principally from distal ICA occlusion. The fates of patients with and without a major arterial occlusive lesion might differ after intravenous (IV) tissue plasminogen activator (alteplase) therapy, because resistance to clot lysis and the fragility of infarcted brain tissue may depend on the patency of the ICA. Rapid evaluation of arterial status in the emergency clinical setting may help predict outcome after alteplase therapy.

Magnetic resonance angiography (MRA) and computed tomographic angiography (CTA) can detect occlusions or severe stenoses of the cervicocephalic arteries supplying the infarcted area in patients with acute stroke,[2,3] as well as intracranial abnormalities with greater sensitivity and specificity, than conventional cerebral angiography.[3,4] Large ischemic lesions on diffusion magnetic resonance

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imaging (MRI) before IV alteplase therapy predict poor outcome in patients with acute ischemic stroke,[5] and diffusion–perfusion mismatch can select patients with remaining salvageable tissue.[6] But MRI takes at least 15 minutes, including equipment arrangement and patient transfer, to generate information, and CTA carries a risk of renal failure and anaphylaxis.

Carotid duplex ultrasonography (US) is another noninvasive tool that can detect major extracranial carotid arterial disease.[7-10] Compared with conventional cerebral angiography, US is not associated with such invasive complications as cerebral and systemic embolism, contrast agent anaphylaxis, acute renal dysfunction, and arterial dissection.[11] Morever, with bedside US, it takes only a few minutes to detect significant occlusive lesions of carotid arteries. US findings can help identify the mechanism and type of ischemic stroke.

We tested the hypothesis that carotid duplex US findings can help predict the outcome and safety of IV alteplase therapy for patients with hyperacute ischemic stroke.

Materials and Methods

We prospectively enrolled all patients with stroke who were admitted to our emergency stroke care unit and received IV alteplase therapy between October 2005 (when this therapy was approved in Japan) and July 2008. Our institution's Ethics Committee approved the research protocol. Patients or their representatives (eg, family members) provided written informed consent for the treatment.

Patient eligibility for IV alteplase therapy was based principally on the inclusion and exclusion criteria applied in the National Institute of Neurological Disorders and Stroke (NINDS) study[12] and in the Japan Alteplase Clinical Trial (J-ACT).[13] Each patient received a single IV dose of 0.6 mg/kg (not exceeding 60 mg) of alteplase, with 10% given as a bolus, followed by a continuous IV infusion of the remainder over 1 hour, in accordance with the Japanese guidelines for IV alteplase therapy based on the J-ACT results.[13,14] As in the NINDS study,[12] the use of antithrombotic agents were prohibited for 24 hours after onset, blood pressure was maintained at <180/105 mm Hg, and neurologic symptoms were monitored.

Clinical data included age and sex; time from symptom onset (or time when the patient last appeared to be normal) to the initiation of IV alteplase therapy; carotid artery US findings before or immediately after the initiation of alteplase therapy; National Institute of Health Stroke Scale (NIHSS) score immediately before (baseline) and 24 hours after alteplase therapy; concomitant diseases; current smoking and drinking habits; imaging data, including hemorrhagic transformation detected by computed tomography (CT) or MRI during hospitalization; stroke subtype according to Trial of Org 10172 in Acute Stroke Treatment (TOAST) criteria;[15] and modified Rankin Scale (mRS) score at day 90. Among concomitant diseases, hypertension was defined as systolic blood pressure ≥140 mm Hg or diastolic blood pressure ≥90 mm Hg before stroke onset or the use of antihypertensive medication. Diabetes was defined as preceding fasting blood glucose ≥126 mg/dL or the use of oral antidiabetic agents or insulin. Hypercholesterolemia was defined as total plasma cholesterol level ≥220 mg/dL or the use of antihypercholesterolemic medication.

Patients underwent US after hospitalization while awaiting the results of blood tests or immediately after starting alteplase therapy. US was performed with a bedside unit (Sonos 5500; Philips Medical Systems, Tokyo, Japan) with a 3- to 11-MHz linear transducer. On US, absent color flow signals on the ICA indicates the occlusion at or proximal to the artery, and absent end-diastolic flow velocity of the ICA indicates intracranial ICA occlusion.[16] Thus, carotid artery occlusion was defined as either of these US findings (Fig 1). Based on the US findings, the patients were divided into 2 groups: those with carotid artery occlusion (designated the CO group) and those without carotid artery occlusion (designated the non-CO group).

Before alteplase therapy, all patients underwent intracranial MRA to serve as the gold standard reference of carotid US findings, unless contraindicated. MRA was performed using the 3-dimensional time-of-flight technique (repetition time/echo time, 35/7.2 msec; 20-degree flip angle) with a 1.5 T system (Magnetom Vision; Siemens, Germany).

Outcomes included early neurologic improvement, defined as a ≥4-point reduction in NIHSS score within the initial 24 hours, and complete independence in activities of daily living (ADL), defined as an mRS score of 0 or 1, at 90 days. To assess long-term independence, patients with a mRS score of ≥2 before stroke onset were excluded. Safety outcomes included any intracranial hemorrhage (ICH) confirmed by head CT or MRI during hospitalization, and symptomatic ICH defined as early ICH with neurologic deterioration corresponding to a ≥1-point increase in the NIHSS score within 36 hours after alteplase therapy.

Statistical Analysis

Sensitivity, specificity, positive predictive value, and negative predictive value for detectng patients with carotid artery occlusion by carotid US were calculated when intracranial MRA findings were used as gold standard. Continuous and categorized variables were compared using the Student *t*-test and the χ^2 test, respectively. Nonparametric independent group comparisons were done using the Mann-Whitney *U*-test. To determine independent clinical variables to predict outcomes, significant variables were analyzed in a logistic regression model, with multivariate adjustments for age, sex, and confounders with an association of $P < .05$ with each outcome in univariate analysis. Statistical significance was established at $P < .05$.

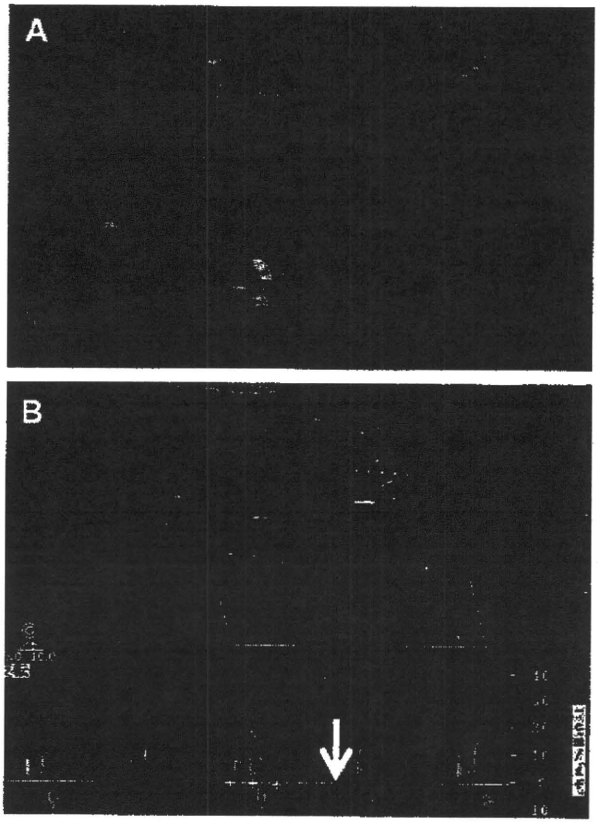

Figure 1. *Typical carotid US findings in ICA occlusion. (A) Absent flow of color in the affected ICA origin in a patient with atherothrombotic extracranial ICA occlusion. (B) Absent end-diastolic flow velocity of affected ICA (arrow) detected by pulsed Doppler US in a patient with distal ICA occlusion.*

Results

A total of 127 patients (89 men, mean age, 73 ± 9 years) were enrolled in the study. In 27 patients, carotid artery occlusion was detected by carotid US before or immediately after alteplase therapy. A total of 110 patients (87%) underwent MRA; 23 were found to have ICA occlusion. Sensitivity, specificity, positive predictive value, and negative predictive value for detect carotid artery occlusion by carotid US were 96%, 97%, 88%, and 99%, respectively. Table 1 summarizes the baseline characteristics and clinical outcomes of the study population. The median baseline NIHSS score was 13 (range, 4-30) and was higher in the CO group than in the non-CO group ($P = .005$). The median duration from symptom onset to IV alteplase therapy was 135 min (range, 50-180 min). US found no evidence of common carotid artery dissection possibly extending from the aortic arch in any patient. This finding, in combination with later examinations, ruled out aortic dissection in all patients.

Cardioembolism was the leading stroke subtype (57%). Atrial fibrillation was more common in the CO group than in the non-CO group. Early neurologic improvement and independence at day 90 were apparently less frequent in the CO group, whereas any ICH was more frequent in the CO group. Two patients in the CO group (7.4%) died within 90 days, one of symptomatic ICH and the other (who had asymptomatic ICH) of severe cerebral herniation due to massive stroke.

We used univariate analysis to test associations of the characteristic variables listed in Table 1 with outcomes (Table 2). Baseline NIHSS ($P = .042$), diabetes mellitus ($P = .049$), and carotid artery occlusion ($P = .039$) were inversely associated with early neurologic improvement. High pretreatment NIHSS score ($P = .015$) and carotid artery occlusion ($P = .002$) were inversely associated with independence at day 90. High baseline NIHSS score ($P = .047$) and carotid artery occlusion ($P = .009$) were associated with any ICH. No variables were significantly associated with symptomatic ICH.

We analyzed the contributing factors to the efficacy and safety outcomes using multivariate adjustment (Table 3). The CO group was independently associated with the absence of early neurologic improvement (odds ratio [OR] = 3.79; 95% confidence interval [CI] = 1.39-11.42; $P = .008$), absence of complete independence at day 90 (mRS score of ≥2: OR = 4.44; 95% CI = 1.38-19.96; $P = .011$), and presence of ICH (OR = 3.11; 95% CI = 1.23-8.48; $P = .016$). Diabetes mellitus (OR = 2.77; 95% CI = 1.03-8.15; $P = .043$) and low NIHSS score (OR = 1.09; 95% CI = 1.02-1.18 per 1-point decrease; $P = .011$) were associated with the absence of early neurologic improvement.

Discussion

Our data indicate that the likelihood of a good outcome was decreased and the likelihood of ICH was increased in stroke patients with US-identified ICA occlusion after IV alteplase therapy. Rapid evaluation using US thus helped predict the effectiveness and safety of alteplase therapy.

Sites of arterial occlusion before alteplase therapy have frequently been identified using transcranial Doppler (TCD) sonography. Recanalization of the ICA after IV alteplase therapy documented on TCD or angiography is reportedly complete in 10% of patients, partial in 16%, and absent in 74%.[17] In addition, terminal ICA occlusion has the least likelihood of recanalization compared with the other types of occlusion (OR = 0.1).[18] Linfante et al[19] found that patients with ICA occlusion have higher NIHSS scores on days 1 and 3 and a lower proportion of recanalization defined by TCD or MRA compared with those with MCA occlusion after alteplase therapy. Consequently, occlusions at the terminal ICA and at a tandem lesion of the ICA and MCA are predictive of poor outcome after alteplase therapy.[18,20] On the other hand, whether carotid US can detect ICA occlusion in the clinical setting of alteplase therapy has not been unequivocally established.

We used carotid US to evaluate the major cerebral arteries because Asian patients with stroke generally do

Table 1. *Baseline characteristics and clinical outcomes*

		US findings	
	Total (n = 127)	CO group (n = 27)	Non-CO group (n = 100)
Characteristic variables			
Female sex	38 (30)	8 (30)	30 (30)
Age, years	73 ± 9	75 ± 8	73 ± 10
Baseline NIHSS score	13 (4-30)	18 (5-24)	12 (4-30)§
Onset to treatment, minutes	135 (50-180)	130 (79-180)	135.5 (50-180)
Hypertension	80 (64)	21 (78)	59 (59)
Diabetes mellitus	24 (19)	5 (19)	19 (19)
Hypercholesterolemia	34 (27)	7 (26)	27 (27)
Atrial fibrillation	58 (46)	17 (63)	41 (41)‡
Current smoking	31 (25)	8 (30)	23 (23)
Alcohol	59 (47)	14 (52)	45 (45)
Stroke subtype			
Large vessel	21 (17)	7 (26)	14 (14)
Cardioembolic	72 (57)	16 (59)	56 (56)
Small vessel	2 (2)	0 (0)	2 (2)
Other	32 (26)	4 (15)	28 (28)
Outcome variables			
Early neurologic improvement*	60 (47)	8 (30)	52 (52)‡
mRS score at 3 months	3 (0-6)	4 (0-6)	2 (0-6)§
Complete independence at 3 months†	44 (35)	3 (11)	41 (41)§
Any intracranial hemorrhage	61 (48)	19 (70)	42 (42)§
Symptomatic intracranial hemorrhage	5 (4)	1 (4)	4 (4)

Values are mean ± standard deviation in age, median (range) in baseline NIHSS score, interval between onset and treatment and mRS score at 3 months, or number (%) in the remaining variables.

*Reduction in NIHSS score of ≥4 points within the initial 24 hours.

†Defined as a mRS score of 0 or 1. Eleven patients with a score ≥2 before stroke onset were excluded.

‡$P < .05$.

§$P < .01$.

not have a sufficient bone window for TCD,[21,22] and obtaining information about arterial occlusion from TCD can be difficult. As an alternative, carotid US can detect intracranial ICA occlusion based on the absence of end-diastolic flow velocity.[16] The accuracy of the diagnosis of carotid occlusion by US is sufficiently high compared with MRA findings. B-mode, color Doppler, and pulsed-wave Doppler carotid US can identify an ICA occlusion in about 5 minutes. The American Heart and Stroke Association recommends completing the initial evaluation and starting medical therapy within 60 minutes of the patient's arrival at the emergency department.[23] Head CT and bedside carotid US imaging can be completed at the emergency department within the 20 minutes or so needed to generate the results of blood tests, including serum chemistry and hemostatic parameters, at our institute.

Another reason for the routine use of carotid US is to rule out aortic dissection extending to the CCA. Concomitant aortic dissection is a conspicuous cause of in-hospital death following IV alteplase therapy in Japan (Japan Stroke Society; http://www.jsts.gr.jp [in Japanese]).

The present study has some limitations. Carotid US cannot provide information about tandem lesions. The incidence of symptomatic ICH was too low to enable an assessment of its relationship with carotid US findings.

In summary, carotid US is a simple tool for detecting ICA occlusion within a few minutes in the emergency clinical setting of hyperacute stroke. Patients with ICA occlusion according to carotid US had worse outcomes and more ICH after IV alteplase therapy. Therefore, rapid non-invasive evaluation of the carotid artery using US might improve the selection of patients likely to benefit from IV alteplase therapy. Although ICA occlusion is a pessimistic sign for success in IV alteplase therapy, patients with such a lesion may still be candidates for this therapy until an alternative therapeutic strategy is established. In the near future, endovascular thrombus retrieval and

Table 2. *Univariate analysis of outcomes*

	Early neurologic improvement*		Complete independence at day 90†		Any ICH		Symptomatic intracranial hemorrhage	
	Present (n = 60)	Absent (n = 67)	Present (n = 44)	Absent (n = 83)	Present (n = 61)	Absent (n = 66)	Present (n = 5)	Absent (n = 122)
Females	19 (32)	19 (28)	11 (25)	27 (33)	20 (33)	18 (27)	2 (40)	36 (30)
Age, years	72 ± 9	75 ± 9	71 ± 8	74 ± 10	72 ± 9	74 ± 10	78 ± 8	73 ± 10
Baseline NIHSS score	13 (5-30)	11 (4-24)‡	11 (4-30)	13 (4-26)‡	14 (4-24)	11 (4-30)‡	15 (12-21)	12 (4-30)
Onset to treatment time	127.5 (50-180)	140 (78-178)	133.5 (50-180)	139 (78-180)	139 (79-180)	133.5 (50-180)	120 (105-143)	136.5 (50-180)
Hypertension	36 (61)	44 (67)	27 (61)	53 (65)	38 (62)	42 (64)	4 (80)	76 (63)
Diabetes mellitus	7 (12)	17 (25)‡	6 (14)	18 (22)	14 (23)	10 (15)	0 (0)	24 (20)
Hyperlipidemia	16 (27)	18 (27)	11 (25)	23 (28)	13 (21)	21 (32)	0 (0)	34 (28)
Atrial fibrillation	26 (44)	32 (48)	16 (36)	42 (51)	30 (49)	28 (42)	4 (80)	54 (45)
Current smoking	10 (17)	21 (31)	9 (21)	22 (27)	16 (26)	15 (23)	1 (20)	30 (25)
Alcohol consumption	30 (51)	29 (43)	22 (51)	37 (45)	28 (46)	31 (47)	1 (20)	58 (48)
Cardioembolic (subtype)	37 (62)	35 (52)	23 (52)	49 (59)	38 (62)	34 (52)	5 (100)	67 (55)
CO group	8 (13)	19 (28)‡	3 (7)	24 (29)§	19 (31)	8 (12)§	1 (20)	26 (21)

Values are mean ± standard deviation in age, median (range) in baseline NIHSS score, and interval between onset and treatment time, or number (%).
*Reduction in NIHSS score of ≥4 points within the initial 24 hours.
†Defined as mRS score of 0 or 1. Eleven patients with a score of ≥2 before stroke onset were excluded.
‡$P < .05$.
§$P < .01$.

Table 3. *Multivariate analysis of outcomes*

	Absence of early neurologic improvement*			mRS score ≥2 at day 90			Any ICH		
	OR	95% CI	*P*	OR	95% CI	*P*	OR	95% CI	*P*
CO group	3.79	1.39-11.42	.008	4.44	1.38-19.96	.011	3.11	1.23-8.48	.016
Diabetes mellitus	2.77	1.03-8.15	.043	–	–	–	–	–	–
Baseline NIHSS score (per 1-point increase)	0.91	0.85-0.98	.011	1.05	0.98-1.13	.144	1.05	0.98-1.12	.165

Adjusted for age, sex, and confounders with an association of $P < .05$ with each outcome in univariate analysis.
Symptomatic intracranial hemorrhage was not tested due to the absence of significantly associated variables in univariate analysis.
*Increase, no change, or decrease in NIHSS score of <4 points within the initial 24 hours.

sonothrombolysis may improve the outcomes of patients with ICA occlusion, at which point this quick screening using US will work well.

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Reduced ischemic brain injury by partial rejuvenation of bone marrow cells in aged rats

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Circulating bone marrow-derived immature cells, including endothelial progenitor cells, have been implicated in homeostasis of the microvasculature. Decreased levels of circulating endothelial progenitor cells, associated with aging and/or cardiovascular risk factors, correlate with poor clinical outcomes in a range of cardiovascular diseases. Herein, we transplanted bone marrow cells from young stroke-prone spontaneously hypertensive rats (SHR-SP) into aged SHR-SP, the latter not exposed to radiation or chemotherapy. Analysis of recipient peripheral blood 28 days after transplantation revealed that 5% of circulating blood cells were of donor origin. Cerebral infarction was induced on day 30 posttransplantation. Animals transplanted with bone marrow from young SHR-SP displayed an increase in density of the microvasculature in the periinfarction zone, reduced ischemic brain damage and improved neurologic function. *In vitro* analysis revealed enhanced activation of endothelial nitric oxide synthase and reduced activation p38 microtubule-associated protein (MAP) kinase, the latter associated with endothelial apoptosis, in cultures exposed to bone marrow-derived mononuclear cells from young animals versus cells from aged counterparts. Our findings indicate that partial rejuvenation of bone marrow from aged rats with cells from young animals enhances the response to ischemic injury, potentially at the level of endothelial/vascular activation, providing insight into a novel approach ameliorate chronic vascular diseases.
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Introduction

Homeostasis of the microcirculation involves a delicate balance between injurious and reparative processes. Repair of the microvasculature has traditionally been considered to result from outgrowth of preexisting vessels to injured areas. More recently, an important contribution of circulating bone marrow-derived immature cells, including endothelial progenitor cells, has been recognized to have a role in the maintenance of the microvasculature, both as a source of endothelial cells (Asahara *et al*, 1997) and growth/angiogenesis factors (Majka *et al*, 2001). Decreased levels of bone marrow-derived circulating endothelial progenitor cells have been demonstrated in patients with cardiovascular risk factors (Hill *et al*, 2003) and correlate with vascular dysfunction (Hill *et al*, 2003) and poor cardiovascular outcomes (Schmidt-Lucke *et al*, 2005; Werner *et al*, 2005). Similarly, we have shown that patients with cerebrovascular disease have decreased circulating bone marrow-derived immature cells, the latter associated with impaired cerebrovascular function (Taguchi *et al*, 2004*a*) and reduced cognition (Taguchi *et al*, 2008, 2009). In contrast, increased levels of bone marrow-derived immature cells are associated with neovascularization of the ischemic brain (Yoshihara *et al*, 2008), and transplanted bone marrow cells improved microcirculatory function in a variety of ischemic organs (Taguchi *et al*, 2003; Tateishi-Yuyama *et al*, 2002). In addition to these clinical observations, studies in experimental ischemia

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demonstrated improved microcirculatory function after transplantation of bone marrow-derived immature cells (Kamihata *et al*, 2001; Taguchi *et al*, 2004*b*).

These clinical and experimental results lead us to hypothesize that 'exhaustion' and/or 'aging' of bone marrow cells in patients with cardiovascular and cerebrovascular diseases may be associated with microvascular dysfunction (Taguchi, 2009). In this study, we used aged stroke-prone spontaneously hypertensive rats (SHR-SP) (Zhang *et al*, 2006) and investigated the effect of partial rejuvenation of their bone marrow with strain-matched bone marrow mononuclear cells from young animals in the setting of experimental stroke.

Materials and methods

All experiments were approved by the Ethics Committee of Ehime University Graduate School of Medicine and the National Cardiovascular Center. All procedures were performed in accordance with the guidelines of the Animal Care Committee of Ehime University Graduate School of Medicine and the National Cardiovascular Center. Quantitative analyses were conducted by investigators who were masked to the experimental protocol and identity of animal, tissue, and experimental conditions pertaining to the animals under study.

Partial Replacement/Rejuvenation of Bone Marrow Cells in Stroke-Prone Spontaneously Hypertensive Rats

Male SHR-SP (SHRSP/Izm; Japan SLC, Hamamatsu, Japan) (Zhang *et al*, 2006), aged 4 to 5 weeks, were used as donors for bone marrow transplantation. Bone marrow cells were obtained from both thigh bones, and mononuclear cells were isolated by density gradient centrifugation using Ficoll (GE Healthcare, Uppsala, Sweden) at 400*g* for 40 minutes according to the manufacturer's protocol. Female SHR-SP, aged around 55 weeks (55 ± 3 weeks) maintained on a high salt diet (OA-2, Japan Clea, Tokyo, Japan) for >40 weeks were recipients of bone marrow cell transplantation. To avoid injury to the microvasculature likely to occur with standard pretreatment for bone marrow transplantation, recipient animals received no radiation or chemotherapy before bone marrow transplantation. Instead, donor bone marrow cells were transplanted by intravenous infusion and direct intrabone marrow injection, the latter to increase transplantation efficiency. Intrabone marrow injection of bone marrow cells has been shown to result in a high seeding efficiency (Inaba *et al*, 2007), and this approach has been used in a clinical trial (Li *et al*, 2007). To obtain the highest seeding efficiency without pretreatment, we used both intravenous and intrabone marrow injection in this study. A dental drill was used to make one hole at each end of the left shin bone, and donor bone marrow mononuclear cells (0.5 mL; 1×10^6 cells/mL suspended in phosphate-buffered saline (PBS)) were injected into one hole whereas suction was applied to the other hole (at the other end of the bone) to remove host bone marrow (Figure 1A). Subsequently, intravenous infusion of the same volume of bone marrow mononuclear cells (0.5 mL; 1×10^6 cells/mL suspended in PBS) was performed using the tail vein. The viability of transplanted cells was 99.2%, evaluated with Trypan Blue staining (Sigma-Aldrich, St Louis, MO, USA). As a control, the same volume of PBS was injected into the bone marrow

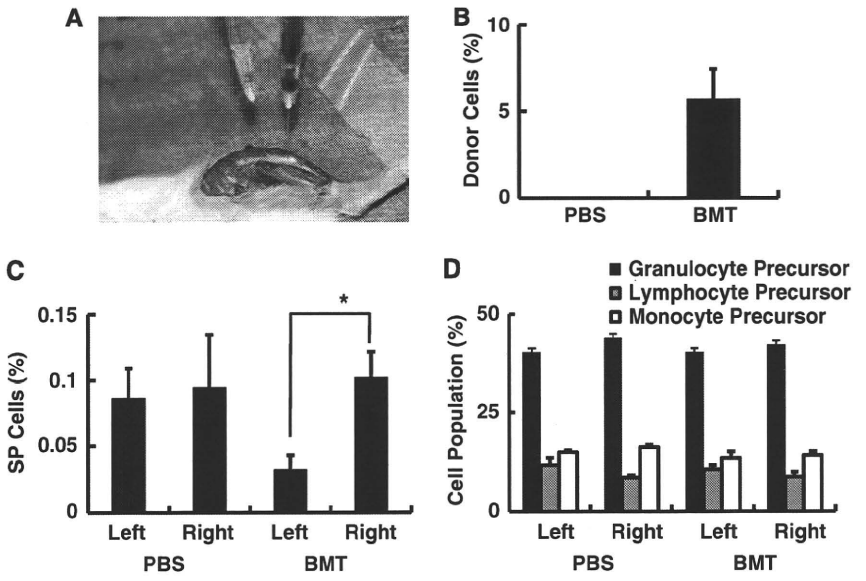

Figure 1 Transplantation of bone marrow from young rats into aged rats. (**A**) Schematic depiction of the intrabone marrow injection. (**B**) Fluorescence *in situ* hybridization (FISH) analysis of peripheral blood on day 28 after bone marrow transplantation. About 5% of circulating cells were donor origin in aged rats transplanted with bone marrow mononuclear cells from strain-matched young rats (BMT, bone marrow transplanted). (**C**) Transplantation of bone marrow from young rats into old rats resulted in a significant reduction in the population of side-population (SP) cells observed in left tibia, compared with right tibia. (**D**) Intrabone marrow injection of bone marrow cells from young animals into older rats did not change the profile of mature cells in the bone marrow. *$P < 0.05$ versus right tibia. $n = 6$, in each group.

and tail vein. Animals were housed in an animal room with a temperature range of 21°C to 23°C and a 12-hour light/dark cycle (light on: 0700 to 1900 hours) for 30 days. The mean arterial blood pressure in each animal was measured using a rat tail manometer–tachometer system (MK-1030, Muromachi Co., Tokyo, Japan) on days 15, 30, and 45 after injection of bone marrow or PBS. No significant difference in blood pressure was observed between groups (data not shown).

Induction of Focal Cerebral Ischemia

On day 28 after transplantation of bone marrow mononuclear cells or PBS injection, focal cerebral ischemia was induced in SHR-SP as described previously (Zhang *et al*, 2006). Briefly, rats were anesthetized with 1.5% halothane in a 4:3 mixture of nitrous oxide and oxygen, and brain temperature was maintained at 37°C ± 0.5°C during the surgery. The left middle cerebral artery above the rhinal fissure and distal to the striate branches was coagulated and cut. After recovery from anesthesia, animals were maintained in an air-conditioned room at ~22°C. On day 30 after induction of stroke, whole brain images were captured with a digital camera system (Olympus, Tokyo, Japan), and the area of intact cortex was measured by NIH image. Sham-operated rats were also studied to evaluate the influence of bone marrow manipulation and induction of stroke on animal survival. The sham group was subjected to an operative procedure on the middle cerebral artery, but no hole was drilled in shin bone to avoid a manipulation that might mobilize bone marrow cells to the systemic circulation.

Assessment of Neurologic Function

To assess cortical function, rats were subjected to behavioral testing using the open field task at 14 days after stroke (Taguchi *et al*, 2004*b*). In this behavioral paradigm, animals were allowed to search freely in a square acrylic box ($60 \times 60\,cm^2$) for 60 minutes. A light source on the ceiling of the enclosure was on during the first 30 minutes (light period) and was turned off during a subsequent 30-minute period. On the *X*- and *Y*-banks of the open field, two infrared beams were mounted 2 cm above the floor, spaced at 10 cm intervals, forming a flip-flop circuit between them. The total number of beam crossings by the animal was counted and scored as traveling behavior. Twelve infrared beams were set 5 cm above the floor, spaced at 3 cm intervals, on the *X*-bank and the total number of beam crossings was counted and scored as rearing behavior. The total count of traveling and rearing behavior was calculated as total locomotion.

To assess spatial learning and memory, rats were subjected to sequential Morris water maze tests at 2 weeks after induction of stroke, as described previously (Zhang *et al*, 2006). Briefly, each test included three trials per day for 4 consecutive days. Rats were allowed to swim until they reached a submerged platform. Then, animals were allowed to remain on the platform for at least 10 seconds. In the event that rats could not find the platform within 90 seconds, they were placed by hand on the platform for 15 seconds and their escape latency was recorded as 90 seconds. The mean latency of finding the invisible platform was measured for individual animals on each day.

Analysis of Peripheral Blood and Bone Marrow Cells

On day 30 after bone marrow transplantation, peripheral blood was analyzed by fluorescence-activated cell sorter (FACS), using anti-CD4, CD8, CD25, CD45RA, NKT, and granulocyte antibodies (all of these antibodies were from BD Bioscience, San Jose, CA, USA), according to the manufacturer's protocol. Peripheral blood was also analyzed to assess the chimera ratio of transplanted (donor):recipient rat blood cells on day 28 after bone marrow transplantation by fluorescence *in situ* hybridization analysis. Briefly, rat-chromosome12-FITC chromosome paint probe was used as a fluorescence *in situ* hybridization control and ratY-Cy3 chromosome paint probe was used to identify donor-derived chromosomal DNA (each probe; Cambio, Cambridge, UK). Fluorescence *in situ* hybridization analysis with Cambio probes was performed according to the manufacturer's instructions as described in http://www.cambio.co.uk. The level of immature cells, side-population (SP) cells (Pearce *et al*, 2007), in bone marrow was evaluated by FACS using Hoechst 33342 (Molecular Probes, Eugene, OR, USA). Briefly, rat bone marrow was obtained from the femurs and tibias of transplanted recipient rats, and single cell suspensions were made by passage of bone marrow through an 18-gauge needle. Bone marrow cells were resuspended at 10^6 cells/mL in prewarmed DMEM containing 2% fetal calf serum, 1 mmol/L Hepes, 100 units/mL penicillin, 100 μg/mL streptomycin, and 5 μg/mL Hoechst 33342. Cell suspensions were then incubated for 90 minutes at 37°C. Resolution of Hoechst populations is highly sensitive to the staining time and Hoechst dye concentration (Watson *et al*, 1985). After Hoechst staining, cells were pelleted and maintained at 4°C before FACS analysis (Becton Dickinson & Co., Mountain View, CA, USA). The level of circulating SP cells was also evaluated, but the number of SP cells in peripheral blood was too small to obtain reproducible data (data not shown).

Analysis of Cytokine Level by Enzyme-Linked Immunosorbent Assay

On day 3 after induction of stroke, serum and tissue samples were obtained from peripheral blood as well as infarcted and periinfarcted cortex, as described in Figure 3A. Proteins were extracted with lysis buffer, containing 1% NP-40 (Sigma-Aldrich), 1% Triton-X (Sigma-Aldrich), and 1 × protease inhibitor cocktail (Sigma-Aldrich). Levels of interleukin (IL)-1β, IL-6, tumor necrosis factor-α, and monocyte chemoattractant protein-1 (MCP-1) were evaluated by enzyme-linked immunosorbent assay (R&D, Minneapolis, MN, USA) according to the manufacturer's protocol.

Immunohistochemistry

Under deep anesthesia with a lethal dose of sodium pentobarbital (0.1 g/kg), the rats were perfused with 4%

paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4). Then, the brains were dissected out and postfixed in the same fixative for 1 day. Coronal sections (20 μm) were prepared using a vibratome (Leica, Wetzlar, Germany) and immunostained according to the standard procedures with antibodies to microtubule-associated protein-2 (MAP-2; Chemicon, Temecula, CA, USA; 1:200), Iba-1 (Wako Pure Chemical Industries, Osaka, Japan; 1:300), Lectin (Invitrogen, Carlsbad, CA, USA; 1:50) and Ki67 (BD Pharmingen, San Jose, CA, USA; dilution 1:20). Infarct volume was evaluated at 30 days after induction of ischemia as described previously (Kasahara *et al*, 2010). Briefly, forebrain samples were sectioned coronally, and a section at each 2 mm interval was stained with anti-MAP-2 antibody. The area staining positively for MAP-2 in each section was measured using a microscopic digital camera system (Keyence, Osaka, Japan). The MAP-2-positive volume of each hemisphere was calculated and percent stroke volume was evaluated by [(contralateral hemisphere volume)−(infarcted hemisphere volume)]/[(contralateral hemisphere volume) × 2] × 100%. Activation of microglia was quantified using anti-Iba-1 antibody as described previously (Taguchi *et al*, 2007) with the following modification. Briefly, the number of Iba-1-positive cells in the anterior cerebral artery area (~0.5 mm from the border of infarction) and contralateral cortex at the exact center of the forebrain section was counted by investigators masked to the experimental protocol (three random fields in each section and the area of each field was 0.12 mm^2). Cerebral vascular density was quantified using anti-Lectin antibody as described previously (Yamahara *et al*, 2008) with the following modification. Briefly, the number of Lectin-positive vascular structures in the anterior cerebral artery area (~0.5 mm from the border of infarction) and contralateral cortex at the exact center of the forebrain section was counted by investigators masked to the experimental protocol (three random fields in each section and the area of each field was 0.12 mm^2). Proliferation of endothelial cells was evaluated with anti-Ki67 antibody, labeled with red fluorescence (Alexa Fluor 555; dilution 1:500) and anti-Lectin antibody, labeled with green fluorescence (Alexa Fluor 488; dilution 1:500). Nuclei were stained with 4′,6-diamino-2-phenylindole (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA).

Wound Healing Model

Backs of SHR-SP were shaved 1 day before creating skin wounds. Skin wounds were placed at the center of back 4 cm caudal from neck. Under deep ketamine anesthesia, wounds were created by performing a full-thickness skin biopsy using an 8-mm punch. Back wounds were left uncovered and no local treatment was applied. To evaluate the healing process, wound diameter was measured 10 days after skin injury. For evaluation of microvasculature, semiquantitative analysis used an angiographic score, according to a modification of a previously described method (Taguchi *et al*, 2003, 2004*b*, 2007). Briefly, pictures of wounds were taken 30 seconds after creation of the wound. Horizontal and vertical lines crossing the exact center of wound were drawn, and the number of vascular structures that crossed each line was counted and the sum of the counts was defined as a semiquantitative angiographic score.

Coculture of Endothelial Cells with Bone Marrow Mononuclear Cells *In Vitro*

Human umbilical vein endothelial cells (HUVECs; Kurabo, Osaka, Japan) were cocultured with aged (>50 weeks old) or young (4 weeks old) SHR-SP bone marrow-derived mononuclear cells. Briefly, 24 hours before coculture, 1×10^6 of HUVEC was plated in six-well plate in HuMedia medium (Kurabo) with 10% fetal bovine serum. One hour before coculture, medium was replaced to HuMedia medium without fetal bovine serum, and 1×10^4 rat-derived bone marrow cells were plated onto the HUVEC. At 2 hours after coculture, plated bone marrow cells were removed, and HUVECs were washed twice with PBS. Activation of HUVEC by bone marrow cells was evaluated based on the level of phosphorylation of endothelial nitric oxide synthase (eNOS), extracellular signal-regulated kinase 1/2 (ERK1/2), p38 and c-Jun NH2-terminal kinase 1/2 (JNK1/2), as described by the manufacturer's protocol (Phospho eNOS (S1177), ERK1/2 (T202 and Y204), p38 (T180 and Y182) and JNK1/2 (T183 and Y185) Flex Set; BD Bioscience). Briefly, total protein was obtained from HUVEC in denaturing buffer containing protease and phosphatase inhibitors, and phosphorylation of eNOS, ERK1/2, p38 and JNK1/2 was evaluated by bead array flow cytometric analysis.

Statistical Analysis

Statistical comparisons among groups were made using the χ^2 test (Figure 2A) or one-way analysis of variance followed by Dunnett test for *post hoc* analysis (Figures 4E and 5K). Individual comparisons were performed using Students' *t*-test. Results are reported as the mean ± standard error. Significance was assumed when $P<0.05$.

Results

Fluorescence *In Situ* Hybridization Analysis After Intrabone Marrow Transplantation

To evaluate transplantation efficiency of intrabone marrow plus intravenous bone marrow transplantation, the chimera ratio of circulating nuclear cells was evaluated by fluorescence *in situ* hybridization analysis on day 28 after cell injection. The number of Y- and X-chromosome positive donor-derived cells and Y-negative and X-positive recipient-derived cells was counted and the ratio of Y-positive donor-derived cells was evaluated. The results indicated that about 5% of circulating cells were Y-chromosome positive in female rats after intrabone marrow plus intravenous transplantation, though

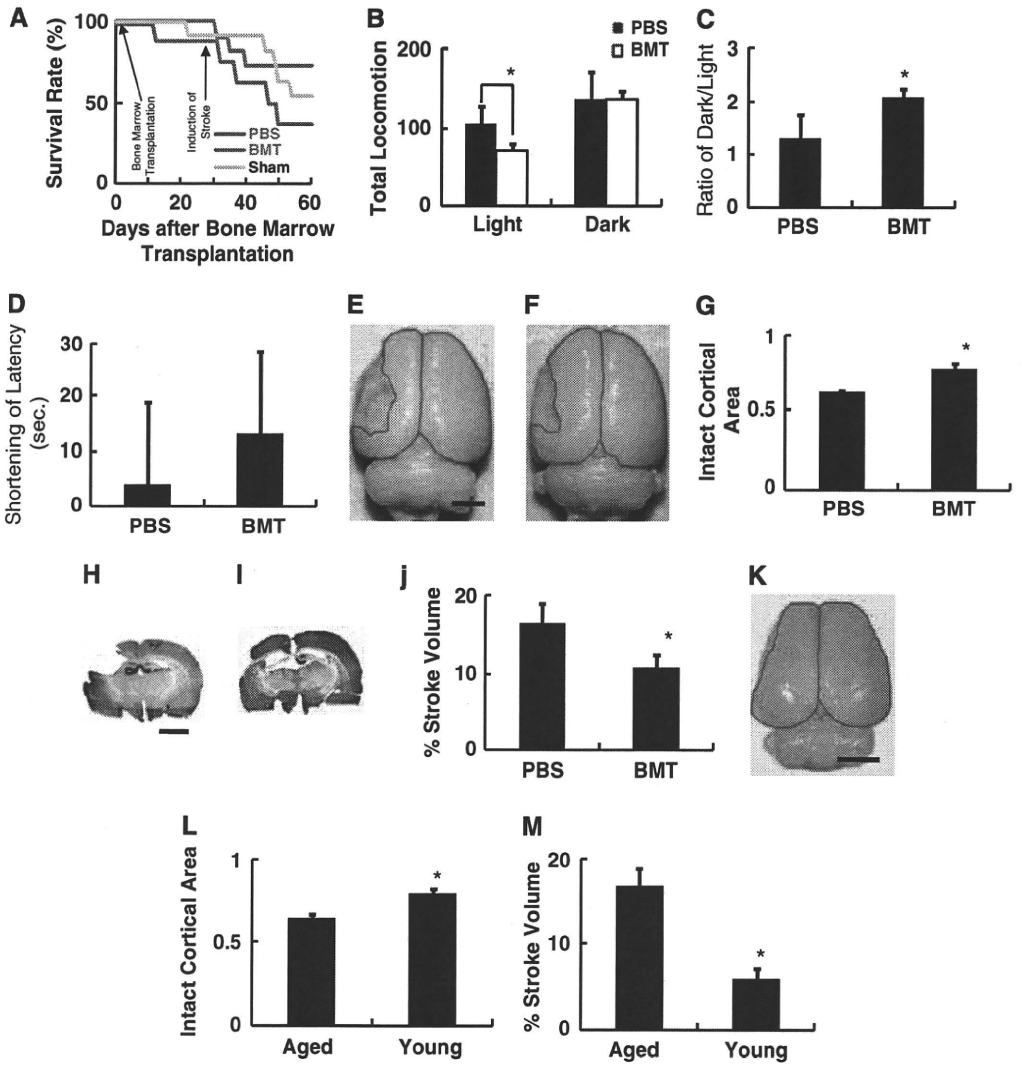

Figure 2 Transplantation of bone marrow from young rats into aged animals reduced ischemic damage in the poststroke period. (**A**) Temporal profile of animal survival for 60 days after bone marrow transplantation. (**B**) Aged animals subject to transplantation with bone marrow cells from young rats (white bar) showed significantly suppressed locomotion in the presence of light on day 14 after induction of stroke, compared with the control group that received phosphate-buffered saline (PBS) alone (black bar). (**C**) Aged animals who underwent transplantation of bone marrow from young animals displayed a significantly improved response in the dark on day 14 after induction of stroke, compared with the PBS-treated control group. (**D**) In contrast to significant improvement of cortical function, mild-to-no significant improvement was observed in memory following the transplantation procedure (day 14 poststroke). (**E–G**) Representative photographs of poststroke brain on day 30 after stroke (**E**: PBS, **F**: bone marrow transplanted (BMT)). The area encircled by red line indicates the intact cortex. Quantitative analysis revealed a significant increase in intact cortical area associated with BMT of young bone marrow into aged animals, compared with PBS-treated controls (**G**). (**H–J**) Representative section of poststroke brain on day 30 after stroke in the PBS (**H**) and BMT (**I**) group. A significant reduction of stroke volume was observed in the BMT group (**J**). (**K–M**) Representative photograph of poststroke brain on day 30 in young stroke-prone spontaneously hypertensive rats (SHR-SP) (**K**). The intact cortical area was significantly larger in young rats, compared with that in aged rats (**L**). A significant increase in stroke volume was observed in aged rats, compared with young rats (**M**). $^*P < 0.05$ versus PBS control (**B**, **C**, **G**, **J**) or aged rat (**L**, **M**). PBS, $n = 8$; BMT, $n = 11$; Sham, $n = 11$ (**A**), PBS, $n = 5$; BMT, $n = 8$ (**B–D**), $n = 5$, in each group (**G**, **J**, **L**, **M**). Scale bar, 0.5 mm (**E**, **H**, **K**).

no Y-chromosome positive cells were observed in PBS-injected control female rats (Figure 1B). To investigate the mature cell population in blood, peripheral blood samples were analyzed to assess a series of parameters including the number of red blood cells, platelets, total white blood cells, CD4-positive T-lymphocytes, CD8-positive T-lymphocytes, NK cells, B cells, and granulocytes. No statistically significant changes were observed comparing peripheral blood of recipients who were transplanted with young bone marrow cells compared with PBS controls (Table 1).

Table 1 Peripheral blood analysis after bone marrow cell transplantation

	PBS	*BMT*	P-*value*
RBC ($\times 10^6/\mu L$)	9.1 ± 0.3	8.8 ± 0.4	0.74
Hb (g/dL)	15.1 ± 0.4	14.2 ± 0.5	0.24
Ht (%)	43 ± 1	41 ± 2	0.26
WBC ($\times 10^2/\mu L$)	8.5 ± 0.3	8.2 ± 0.6	0.74
Granulocyte (%)	34.8 ± 2.5	33.7 ± 2.8	0.77
Lymphocyte (%)	55.9 ± 3.0	47.8 ± 2.2	0.06
CD4+ T cell (%)	10.5 ± 2.6	6.2 ± 2.0	0.24
CD8+ T cell (%)	4.9 ± 1.7	2.4 ± 0.7	0.19
B cell (%)	3.5 ± 0.6	2.7 ± 0.5	0.36
NK cell (%)	4.5 ± 0.4	4.0 ± 0.7	0.53
Monocyte (%)	12.6 ± 2.5	16.7 ± 2.7	0.29
Platelet ($\times 10^4/\mu L$)	41 ± 3	34 ± 4	0.16

BMT, bone marrow transplanted; PBS, phosphate-buffered saline; RBC, red blood cell; WBC, white blood cell.

To analyze the cell population at the site of intrabone marrow injection, the number of SP cells in the shin bone was evaluated. The SP cells are known to include a population of immature cells of hematopoietic lineage that significantly increase with aging in bone marrow (Pearce *et al*, 2007). Consistent with the latter, a significant reduction in the percent of SP cells was observed in shin bone after transplantation of young bone marrow (left) compared with the nontransplanted side (right) (Figure 1C). In contrast, there was no significant difference observed between the two sides in animals subject to PBS injection (Figure 1C). The population of mature hematopoietic cells in bone marrow was also investigated, but no significant differences were observed between the left and right shin bones in rats subject to intrabone marrow plus intravenous injection of young bone marrow cells versus PBS (Figure 1D).

Intrabone Marrow Injection of Young Bone Marrow and the Survival Rate of Aged Rats

The mean lifespan of SHR-SP has been shown to be significantly shorter than that of their wild-type counterparts (Brandle *et al*, 1997). Consistent with this observation, by day 30 after bone marrow or PBS transplantation, 13% and 10% of rats in PBS-treated and nontreated sham-operated group had expired, respectively. In contrast, no deaths were observed in rats transplanted with young bone marrow cells. On day 30 after transplantation of young bone marrow cells or injection of PBS, cerebral ischemia was induced. By day 60, a total of 63%, 27%, and 45% of rats had expired in the PBS, bone marrow transplanted and sham-operated groups, respectively (Figure 2A). There was no significant difference in survival between groups ($P=0.31$). Individual comparisons between each group using the χ^2 test also did not show statistically significant differences, including PBS versus bone marrow transplanted groups ($P=0.12$).

Intrabone Marrow Injection of Young Bone Marrow Reduced Cortical Brain Damage After Stroke

Cerebral ischemia in a rodent model induced by ligation of the middle cerebral artery at a site distal to the striatal branches mainly causes infarction of the cerebral cortex (Zhang *et al*, 2006). Dysfunction of the cortex is closely linked to disinhibition of behavior (Farkas *et al*, 2003). On day 14 after induction of stroke, cortical function was evaluated using the open field task. Compared with PBS-injected rats, significant improvement of behavior, as characterized by suppression of locomotion in the presence of light, was observed in rats after transplantation with bone marrow from young animals (Figure 2B). Analysis of the response of animals placed in the dark also revealed a significant improvement in rats after bone marrow transplantation (Figure 2C).

Next, spatial learning/memory was evaluated using a water maze assay. The change in mean time to escape the submerged platform was evaluated; only a nonsignificant trend favoring improvement was observed in the group subjected to bone marrow transplantation (Figure 2D). The apparent discrepancy between functional recovery (locomotion) and cortical function/learning (water maze) might reflect variations in learning capacity in aged rats compared with a younger group of SHR-SP animals (Meaney *et al*, 1995).

On day 30 after induction of stroke, images of whole brain were captured to evaluate the extent of cortical damage/recovery morphologically. Compared with PBS controls (Figure 2E), significant reduction of ischemic cortical damage was observed in animals subject to bone marrow transplants (Figures 2F and 2G). To confirm the effect of transplanting young bone marrow cells into aged rats on reduction of brain damage poststroke, sequential brain sections were prepared and stroke volume was evaluated with MAP-2 staining. Compared with PBS controls (Figure 2H), significant reduction of stroke volume was observed in animals subject to bone marrow transplantation (Figures 2I and 2J). It is notable that stroke area was limited at the level of the cortex in rats who had received bone marrow transplants, in contrast to controls in which ischemic damage expanded to reach the lateral ventricle.

To evaluate the effect of aging and rejuvenation of the bone marrow with cells from young animals on the size of cerebral infarcts, cerebral infarction was induced in 8-week-old SHR-SP (Figure 2K). Quantitative analysis revealed a significant reduction in the area of infarction in young rats, compared with aged (>50 weeks; Figure 2L). The reduction in stroke

volume was confirmed by sequential sections with MAP-2 staining (Figure 2M).

Changes in Expression of Proinflammatory Cytokines After Intrabone Marrow Injection of Bone Marrow Cells from Young Animals into Old Stroke-Prone Spontaneously Hypertensive Rats

To investigate possible causes of apparent reduction in cortical damage observed in rats transplanted with bone marrow cells from young animals, the level of inflammatory cytokines, including IL-1β, IL-6, tumor necrosis factor-α, and MCP-1, was evaluated 3 days after stroke. The areas from which tissue samples were harvested are shown in Figure 3A. Although functions of each inflammatory cytokine are multiple, increased levels of IL-1β after stroke have been associated with mainly negative effects, including inflammation, apoptosis, and edema (Holmin and Mathiesen, 2000). Similarly, MCP-1 has been ascribed a principally negative function (Chen *et al*, 2003). In contrast, tumor necrosis factor-α has been shown to have both neuroprotective (Sullivan *et al*, 1999) and neurotoxic effects (Yang *et al*, 1998). The IL-6 has been suggested to display principally positive effects (Loddick *et al*, 1998). In the infarcted cortex, a significant increase in levels of IL-1β and MCP-1 was observed in rats subject to bone marrow transplantation compared with PBS-treated controls (Figure 3B). In contrast, there was no significant change observed in levels of IL-6 and tumor necrosis factor-α. Similarly, increased levels of IL-1β and MCP-1 were observed in rats subjected to bone marrow transplantation when the periinfarcted cortex was studied (Figure 3C). The increase in levels of the latter cytokines in the infarcted and periinfarcted cortex and serum, contrast with a decrease of IL-6 levels in serum of rats subject to transplantation (Figure 3D; there was no significant change in IL-6 levels in the infarcted and periinfarcted cortex). These results suggest that modulation in expression of inflammatory cytokines (i.e., favoring recovery/decreased inflammatory profile) did not occur, and, thus, is not likely to explain the beneficial effect of the response to cerebral ischemia observed in older SHR-SP transplanted with bone marrow cells from young SHR-SP.

Intrabone Marrow Injection of Bone Marrow Cells from Young Rats into Old Stroke-Prone Spontaneously Hypertensive Rats Animals Does Not Affect the Host Response to Cutaneous Wounding

To more generally address the issue of the host reparative response in old SHR-SP subject to transplantation of bone marrow cells from young animals, we used a model of cutaneous wound repair. We

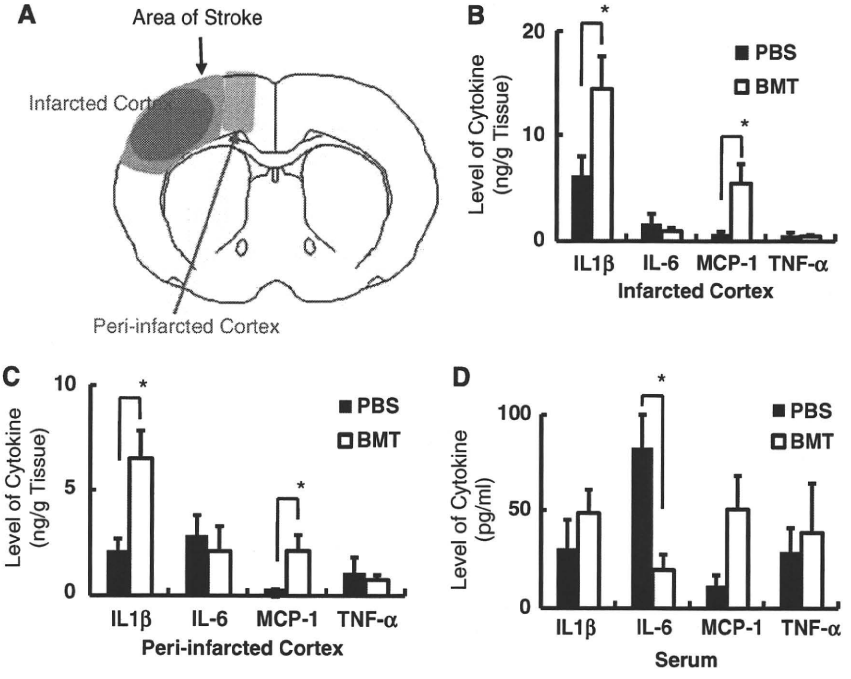

Figure 3 Profile of inflammatory cytokines in poststroke brain. (**A**) Schematic of the brain area from which tissue samples were harvested. (**B**) A significant increase in interleukin (IL)-1β and monocyte chemoattractant protein-1 (MCP-1) was observed following bone marrow transplanted (BMT) of bone marrow from young animals into aged animals in the infarcted cortex on day 3 after induction of stroke. (**C**) Similarly, a significant increase in IL-1β and MCP-1 was observed following BMT of bone marrow from young animals to aged animals in the periinfarcted cortex. (**D**) In contrast, a significant decrease in serum levels of IL-6 was observed in aged animals subject to BMT with bone marrow from young animals. *$P < 0.05$ versus phosphate-buffered saline (PBS) control. $n = 5$, in each group.