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Intravenous Recombinant Tissue Plasminogen Activator Therapy for Stroke Patients Receiving Maintenance Hemodialysis: The Stroke Acute Management with Urgent Risk-Factor Assessment and Improvement (SAMURAI) rt-PA Registry

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Key Words

Acute ischemic stroke · Cerebral infarction · Chronic kidney disease · End-stage renal disease · Hemodialysis · Renal dysfunction · rt-PA · Thrombolysis

Abstract

Background: To examine the therapeutic effect of intravenous recombinant tissue plasminogen activator (rt-PA) therapy for stroke patients receiving maintenance hemodialysis (HD). ***Methods:*** Of 600 stroke patients receiving intravenous rt-PA using 0.6 mg/kg alteplase who were enrolled in a multicenter observational study in Japan, 4 patients (3 men, 64–77 years old) on maintenance HD were studied. ***Results:*** The primary kidney disease requiring HD was glomerulonephritis in 2 patients, diabetic nephropathy in 1, and undetermined in 1. The duration of HD ranged between 1.2 and 28 years. Three patients developed stroke on the day of HD, including 1 during HD and another just after HD. All patients had stroke in the carotid arterial territory. Pretreatment NIH Stroke Scale scores ranged between 4 and 20, and decreased by 2–5 points at 7 days. One patient needed intravenous antihypertensive therapy before rt-PA; he developed an ectopic cortical hematoma and intraventricular hemorrhage after rt-PA. The other 3 did not develop hemorrhagic complications. The modified Rankin Scale score at 3 months was 0 in 1 patient, 2 in 2 patients, and 4 in 1 patient. ***Conclusions:*** rt-PA therapy for stroke patients receiving maintenance HD might improve the stroke outcome. Ectopic hematoma was a unique complication in our case series.

Introduction

Patients receiving hemodialysis (HD) have a higher risk of stroke than the general population [1], and they often develop stroke during or just after HD while they remain in the clinic [2]. Thus, HD patients might have a high opportunity to receive urgent therapies for stroke, including intravenous (IV) recombinant tissue plasminogen activator (rt-PA). HD itself is not a contraindication to IV rt-PA in several guidelines, but heparinization is. In addition, severe renal damage appears to affect the outcome after rt-PA [3, 4].

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Table 1. Baseline characteristics and physiological and laboratory data on admission

	Patient 1 female	Patient 2 male	Patient 3 male	Patient 4 male
Age, years	74	77	68	64
Body mass index	17.6	21.1	20.3	27.9
Primary kidney disease	glomerulonephritis	undetermined	diabetic nephropathy	glomerulonephritis
Duration of hemodialysis, years	28	2	1.2	24
Stage of hypertension [13]	high normal	stage I	stage I	stage II
Other vascular risk factors	atrial fibrillation[1]	sick sinus syndrome	diabetes mellitus	–
Vascular comorbidities	MI, silent brain infarct	angina pectoris	MI	–
Other comorbidities	hepatitis C virus carrier, hyperparathyroidism	–	meningioma (resected)	gastric cancer (resected)
Premorbid modified Rankin Scale score	0	0	0	0
Prior medication				
Antithrombotics	aspirin	aspirin	none	none
Antihypertensives (vasodilator)	ISDN	torasemide, ISDN	none	nifedipine, limaprost
Antidiabetics	none	none	insulin	none
Physiological/laboratory data on admission				
Blood pressure, mm Hg	202/83	165/81	150/86	218/98
Platelet count, /μl	254,000	175,000	140,000	124,000
Hemoglobin, g/dl	12.1	12.9	10.6	10.6
Prothrombin time (INR)	1.13	0.89	1.10	0.90
Activated partial thromboplastin time, s	43.5	26	36.4	32
Blood urea nitrogen, mmol/l	3.9	22.8	12.1	11.8
Creatinine, μmol/l	230	919	327	415
Blood glucose, mmol/l	5.7	10.5	12.7	5.0
Hemoglobin A_{1c}, %	4.3	5.3	5.9	not measured
Total cholesterol, mmol/l	3.29	4.12	4.17	4.25
Triglyceride, mmol/l	0.59	1.24	1.50	0.64
HDL cholesterol, mmol/l	1.14	1.48	0.83	1.40
LDL cholesterol, mmol/l	1.89	2.07	2.64	2.41

INR = International normalized ratio; ISDN = isosorbide dinitrate; MI = myocardial infarction.
[1] Identified during acute hospitalization after stroke onset.

We have reported the effects of IV rt-PA given to stroke patients with renal dysfunction using the Stroke Acute Management with Urgent Risk-factor Assessment and Improvement (SAMURAI) rt-PA Registry [4]. Reduced estimated glomerular filtration rate <60 ml/min/1.73 m^2 on admission was independently associated with intracerebral hemorrhage (ICH) within 36 h after rt-PA and unfavorable functional outcome or death at 3 months. The results suggest that end-stage renal disease (ESRD) is also associated with poor outcome after rt-PA, although, to the best of our knowledge, this issue has never been examined.

The aim of this study was to determine the effect of IV rt-PA therapy in stroke patients on maintenance HD using the same registry.

Patients and Methods

The SAMURAI rt-PA Registry had a multicenter, hospital-based, retrospective, observational, cohort design [4–6]. A total of 600 consecutive patients with acute ischemic stroke receiving alteplase at 0.6 mg/kg (the recommended dose in Japanese guidelines and the approved labeling) from October 2005 through July 2008 were registered. From the registry, ESRD patients receiving maintenance HD or peritoneal dialysis were studied. The local ethics committee approved the research protocol. Baseline characteristics, physiological and laboratory data on admission, stroke features, and outcomes were assessed for each patient. Diffusion-weighted MRI (DWI) and MRA were performed before rt-PA infusion in addition to head CT. Early ischemic change was assessed using the Alberta Stroke Program Early CT Score (ASPECTS) [6].

Results

Of the 600 patients, none were on peritoneal dialysis and 4 (0.7%, 3 men, 64–77 years old) were undergoing maintenance HD before IV rt-PA therapy. These 4 patients were studied.

Baseline characteristics and physiological and laboratory data on admission are listed in table 1. In brief, the primary kidney disease responsible for HD was glomeru-

Table 2. Stroke features and outcomes

	Patient 1	Patient 2	Patient 3	Patient 4
Timing of stroke onset	just after HD	non-HD day	2 h after HD	during HD
Major neurological signs	aphasia, unilateral spatial neglect, right hemiparesis	unilateral spatial neglect, left hemiparesis	aphasia, right facial palsy	aphasia
ASPECTS on CT	10	9	10	10
ASPECTS on DWI	9	8	9	10
Site of arterial occlusion	M2	ICA	undetectable	undetectable
Stroke territory	left carotid	right carotid	left carotid	left carotid
Stroke etiology	cardioembolism	cardioembolism	undetermined	undetermined
Onset to rt-PA time, min	130	139	150	166
Pre-rt-PA antihypertensives	none	none	none	IV nicardipine
Antithrombotic therapy after rt-PA	IV unfractionated heparin 24 h after rtPA followed by warfarin	IV argatroban 24 h after rtPA followed by warfarin	IV unfractionated heparin 24 h after rtPA followed by aspirin	IV unfractionated heparin 48 h after rtPA followed by aspirin
Timing of restarting HD after rt-PA	20 h later	20 h later	2 days later	22 h later
Intracerebral hemorrhage during acute stage	absent	absent	absent	present (see fig. 2)
NIH stroke scale score				
Baseline	20	13	11	4
24 h after rt-PA	18	11	5	5
7 days after rt-PA	18	9	6	2
Modified Rankin Scale score at 3 months	4	2	2	0

ICA = Internal carotid artery.

lonephritis in 2 patients, diabetic nephropathy in 1, and undetermined in 1. The duration of HD ranged between 1.2 and 28 years. All patients had hypertension, and 2 were taking aspirin prior to stroke. Stroke features and outcomes are listed in table 2. One patient developed stroke during HD and another just after HD. All patients had stroke in the carotid arterial territory; 2 were due to cardioembolism and 2 were of undetermined mechanisms. In the latter 2, emboligenic diseases were not identified using transesophageal echocardiography and Holter ECG. For patient 4, hemodialytic procedure by itself may be a possible cause of stroke since he developed stroke during HD. One patient needed IV antihypertensive therapy just before rt-PA. Pretreatment NIH Stroke Scale scores ranged between 4 and 20 and decreased by 2–5 points at 7 days. No patients showed neurological deterioration. The modified Rankin Scale score at 3 months was 0 in 1 patient, 2 in 2 patients, and 4 in 1 patient.

Early ischemic changes on baseline DWI are shown in figure 1. Early ischemic changes were found in the left insular and frontal cortices in patient 1, the right basal ganglia and corona radiata extending to the insular cortex in patient 2, and the left basal ganglia and corona radiata in patient 3. DWI-ASPECTS in these patients ranged between 8 and 9. In patient 4, ischemic changes were not identified on the baseline DWI, and they were later detected as tiny scattered infarcts in the left cortex. This patient developed transient headache and vomited once, 1 h after rt-PA; CT revealed an ectopic hematoma in the left temporal lobe with the left intraventricular hemorrhage. This patient had IV heparin 48 h after rt-PA, and the hematoma no longer grew after that. The other 3 patients did not develop any intracranial or systemic hemorrhagic complications.

Discussion

In this observational study, 4 stroke patients with HD receiving IV rt-PA were reported. The major finding was that 3 patients had functional independence (modified Rankin Scale score ≤ 2) at 3 months, although ICH with transient headache occurred in 1 of these 3.

Stroke patients with ESRD are at a disadvantage for IV rt-PA for several reasons [7–9]. First, advanced diabetes, which is known to be associated with poor outcome after IV rt-PA, is frequent in ESRD patients. Second, ESRD patients often have hypertension resistance to

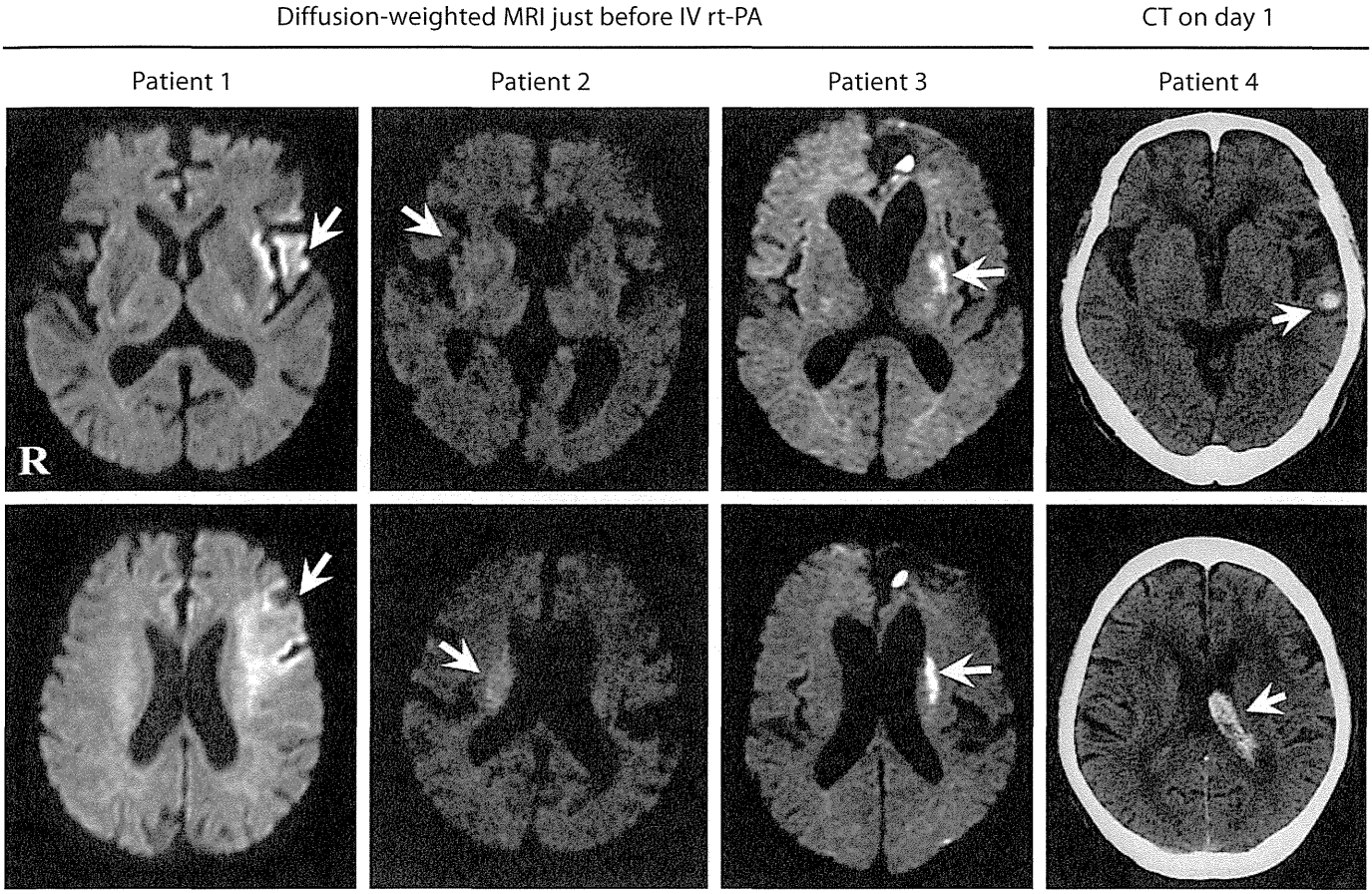

Fig. 1. DWI just before IV rt-PA therapy in patients 1–3, and CT on the day of thrombolysis in patient 4. Arrows show early ischemic changes or ectopic hemorrhage.

antihypertensives and other vascular risk factors and vascular comorbidities as predictors for a poor outcome. Third, blood surface interactions during HD lead to impairment of platelet function and a decrease in platelet number.

Another major disadvantage of ESRD patients is their high risk of ICH. Renal dysfunction is a predictor for hemorrhagic transformation in acute ischemic stroke with or without thrombolysis, presumably partly due to endothelial dysfunction related to renal dysfunction [4, 10]. Previous studies reported a relatively high percentage of ICH among total stroke in ESRD patients [1, 2]. In addition, HD is generally given three times per week using heparin as an anticoagulant, and the activated partial thromboplastin time often exceeds the normal range. A unique finding of the present study was the ectopic hematoma after rt-PA in patient 4. Since 19–35% of patients receiving HD had cerebral microbleeds documented on T_2*-weighted, gradient echo MRI [11, 12], such microbleeds might have grown to be an overt hematoma in this patient. Receiving rt-PA soon after stopping HD (although activated partial thromboplastin time returned to the normal range) and the high baseline blood pressure that required IV antihypertensive therapy may have triggered this ICH; the coexistence of such conditions may be a contraindication to rt-PA.

In spite of several disadvantages, 3 of the present 4 ESRD patients had functional independence 3 months after rt-PA. Since the study population was small, the efficacy and risk of IV rt-PA in ESRD patients could not be determined from this study alone. However, IV rt-PA does appear to be effective for some ESRD patients. A comparison between the patient having a poor outcome and the other patients suggests that initial neurological severity is a good predictor of outcome after rt-PA, as in general stroke patients. Moreover, these 4 patients, in-

cluding the 1 with a poor outcome, had relatively mild early ischemic changes.

Since thrombolysis for ESRD patients has been understudied, one often hesitates to use rt-PA for ESRD patients with hyperacute stroke. Furthermore, one might wonder if HD within 24 h of rt-PA is safe or not. The strength of this study is to report that IV rt-PA is a feasible strategy for ESRD patients for the first time as far as we know.

This study's limitations included its retrospective, observational design, the small number of ESRD patients, and the lack of data on patients who did not receive thrombolysis for stroke. Another limitation was that the present results, which were based on low-dose alteplase, may not be applicable to the regular-dose therapy (0.9 mg/kg).

Appendix

Stroke Acute Management with Urgent Risk-Factor Assessment and Improvement (SAMURAI) Study Investigators:

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Central Study Office: M. Koga, M. Naganuma, and H. Kanai, National Cerebral and Cardiovascular Center.

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Disclosure Statement

There are no conflicts of interest to disclose.

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High level of plasma adiponectin in acute stroke patients is associated with stroke mortality

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ABSTRACT

We examined the association between plasma adiponectin (ADPN) levels and cardiovascular mortality in acute stroke patients. We enrolled 552 consecutive acute stroke patients. Measurements were made at baseline and the patients were followed prospectively. The primary endpoint was cardiovascular (stroke or ischemic heart disease) death and the secondary endpoint was all-cause death. During the median follow-up period of 17 months, 39 patients died, 15 being due to stroke. No patients died of ischemic heart disease. After adjustment for age, sex, presence of hypertension, diabetes mellitus, and hyperlipidemia, the highest tertile of ADPN level (>11.7 μg/ml) was associated with stroke mortality (hazard ratio: 6.55, 95% confidence interval: 1.73–24.8), but not with all-cause mortality (hazard ratio: 1.89, 95% confidence interval: 0.95–3.77). High levels of plasma ADPN can be a predictor of stroke mortality during the 17 months following an episode of acute stroke in patients.

1. Introduction

Adiponectin (ADPN) is a peptide hormone secreted by adipocytes, shown to have a number of beneficial effects, such as anti-atherosclerosis and anti-inflammatory properties, and improvement of insulin resistance in the general population [1]. A number of reports suggested that hypoadiponectinemia is associated with high incidence of coronary artery disease [2] and of ischemic stroke [3]. Hypoadiponectinemia in patients with ischemic stroke was associated with long-term mortality [4]. In an animal model, ADPN exhibited vascular protection and prevention of brain damage after acute ischemic injuries, which were mediated by an endothelial nitric oxide synthase-dependent mechanism [5]. In these studies, it was suggested that a low level of ADPN is a cardiovascular risk factor and the secretion or action of ADPN might reduce the risk of cardiovascular disease (CVD).

On the contrary, several recent studies demonstrated that a high level of ADPN was associated with risk of CVD events and mortality in the general population [6], as well as in patients with chronic heart failure [7–9], coronary artery disease [10], or chronic kidney disease [11]. ADPN was shown to improve insulin resistance, and administration of ADPN decreased body weight in animal model [12]. It is suggested that high levels of ADPN might act to promote wasting and cause body weight loss, which is associated with poor prognosis in CVD patients.

Thus, it is unclear whether ADPN plays a beneficial or a harmful role in CVD. The purpose of the present study was to examine the association between plasma ADPN levels and cardiovascular mortality in acute stroke patients.

2. Methods

2.1. Subjects

This was a single-center hospital-based prospective study that was approved by the Institutional Research and Ethics Committee of the National Cardiovascular Center, Japan. We registered 569 patients who were admitted to our Stroke Care Unit within 7 days of stroke onset from April 1, 2005 to August 31, 2007. Not counted in this group were patients with subarachnoid hemorrhage due to a ruptured aneurysm and those with massive brain hemorrhage requiring neurosurgical treatment. Because informed consent was not obtained from 17 patients, 552 patients (men/women = 367/185, median age, 71 years) were actually enrolled in the present study.

2.2. Baseline assessment

Brain CT, carotid ultrasonography, and ECG were performed for all patients at the time of admission. The cervico-cephalic arteries of all patients with ischemic stroke, who did not have an implanted pacemaker, were evaluated with magnetic resonance angiography in addition to carotid ultrasonography. Two-dimensional echocardiography

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was done to investigate a potential embolic source in patients with ischemic stroke. Morning blood samples were collected after overnight fasting for measurement of glucose, lipid levels, and ADPN, 2 weeks after the stroke onset. For 91 patients in this study, we confirmed that there were no significant differences between serum ADPN levels on admission and 2 weeks after the stroke onset (data not shown). Plasma glucose was measured by the glucose-oxidase method and plasma ADPN level was measured by enzyme-linked immunosorbent assay. Triglycerides, total cholesterol, low density lipoprotein cholesterol, high density lipoprotein cholesterol, and creatinine were measured enzymatically. Renal function was assessed by the estimated glomerular filtration rate (eGFR) formula using the equations for the Japanese population [13] from serum creatinine: eGFR (ml/min/1.73 m^2) = $194 \times (\text{serum creatinine})^{-1.094} \times (\text{age})^{-0.287} \times 0.739$ (for woman). Blood pressure was measured in all patients before discharge.

The diagnosis of stroke subtypes, such as atherothrombotic brain infarction (n = 90), lacunar infarction (n = 82), cardioembolic infarction (n = 130), other types of infarction (n = 123), and brain hemorrhage (n = 127), was made as previously described [14]. A diagnosis of atherothrombotic brain infarction was based on the presence of focal brain infarct(s) in the collection of evidence for occlusive lesions in the large cervical and intracranial arteries (either occlusion, ≥50% stenosis of the lumen diameter, or ulceration) determined by the clinical data. The diagnosis of lacunar infarction was made when a typical clinical syndrome was associated with a small infarct, <15 mm in diameter on CT, restricted to the territory of a perforating artery, and when no evidence of adjacent major artery occlusion or severe stenosis was found. Cardioembolic infarction was clinically diagnosed as described elsewhere [15]. If the patient had a combination of the above etiologies, or had undetermined etiologies (n = 72), if the stroke had other causes, such as arterial dissection, cerebral venous thrombosis (determined etiologies, n = 51), the index stroke was categorized to other types of infarction. The diagnosis of brain hemorrhage was based on CT findings.

National Institutes of Health Stroke Scale (NIHSS) scores were assessed on admission.

2.3. Patient follow-up

Patients were registered on the admission day. Information on vital status after the discharge from our hospital was obtained from medical charts of out-patient clinics or with telephone interview. All patients were followed up for at least 3 months until September, 2008. The primary endpoint was cardiovascular death (stroke and ischemic heart disease) and secondary endpoint was all-cause death.

2.4. Statistical analysis

Statistical analyses were performed using the SPSS 16.0J statistical package (SPSS, Inc., 2007). A value of $p<0.05$ was considered statistically significant.

ADPN levels were divided into tertiles (<6.8, 6.8–11.7, and >11.7 μg/ml). To determine the differences in clinical characteristics among the three groups of plasma ADPN levels or two groups of survivors and the dead, the χ^2 test, Student's t-test, Mann–Whitney U-test, ANOVA with Scheffe's F-test was used as appropriate. The correlations between plasma ADPN level and each cardiovascular risk factor were examined by a single regression analysis. Survival time was calculated from the date of admission to date of death. Survival rate was estimated by the Kaplan–Meier method and compared among the 3 groups by the log-rank test. The independent contribution of each factor to fatal events was estimated by the Cox proportional hazards models. Hazard ratios (HRs) for the incidence of fatal events were evaluated for to the highest tertile of ADPN level (>11.7 μg/ml) against the middle plus the lowest tertiles (≤11.7 μg/ml). Clinical covariates with the presence of hypertension, diabetes mellitus, and hyperlipidemia, were entered into the Cox proportional hazards models to adjust for potential confounders (Model 2). Subsequently, body mass index, eGFR, and NIHSS score on admission were added to covariates (Model 3).

3. Results

Median follow up period of this study was 17 months (range: 1–42 months). Plasma ADPN level of each stroke subtype were as follows: 9.3 ± 6.3 μg/ml in patients with atherothrombotic infarction, 9.0 ± 5.9 μg/ml in patients with lacunar infarction, 14.4 ± 11.1 μg/ml in patients with cardioembolic infarction, 10.5 ± 6.5 μg/ml in patients with other types of infarction, and 11.8 ± 8.6 μg/ml in patients with brain hemorrhage. Mean plasma ADPN level of patients with cerebral thrombosis (atherothrombotic infarction and lacunar infarction) was 9.2 ± 6.1 μg/ml, which was significantly lower than that in patients with cardioembolic infarction (14.4 ± 11.1 μg/ml, $p<0.001$).

Age, gender, body mass index, eGFR, frequencies of current smoker, diabetes, hyperlipidemia, median NIHSS score on admission, and the distribution of stroke subtypes were significantly different among the 3 levels of plasma ADPN (Table 1). In the lowest tertile, the larger number of younger patients and males was contained, and body mass index and eGFR were higher than those in the other tertiles. There were higher frequencies of current smokers, diabetes, and hyperlipidemia in the lowest tertile, and median NIHSS score on admission in the highest tertile was higher than those in the other 2 groups (Table 1). Plasma ADPN levels were significantly positively correlated with age ($r = 0.360$, $p<0.001$), HDL cholesterol ($r = 0.442$, $p<0.001$), and NIHSS score on admission ($r = 0.161$, $p<0.001$), and were negatively correlated with body mass index ($r = -0.346$, $p<0.001$), triglycerides ($r = -0.307$, $p<0.001$), and eGFR ($r = -0.203$, $p<0.001$).

From a total of 552 patients, 4 patients dropped out during the follow up period; 1 patient with atherothrombotic brain infarction, 1 patient with lacunar infarction, and 2 patients with other types of infarction. From the remaining 548 patients, 39 patients died, of which 15 patients died of stroke (3 patients died during hospitalization) during the median follow-up period of 17 months. No patients died of ischemic heart disease. Other causes of death included heart

Table 1
Patient characteristics by tertiles of plasma ADPN.

Adiponectin level	Low (n = 184)	Middle (n = 183)	High (n = 181)	p
Age (years)	66 ± 11	71 ± 11	76 ± 10	<0.001
Male (n)	151	124	88	<0.001
Current smoker (n)	64	47	35	0.004
Ischemic heart disease (n)	21	16	25	0.311
Body mass index (kg/m^2)	24.5 ± 3.5	23.4 ± 3.4	21.2 ± 3.5	<0.001
Hypertension	150	140	148	0.366
Diabetes mellitus	60	55	36	0.016
Hyperlipidemia	77	62	52	0.030
Estimated GFR (ml/min/1.73 m^2)	76.2 ± 19.3	75.6 ± 22.8	65.7 ± 27.2	<0.001
Median NIHSS score on admission	5 (range: 0–28)	5 (range: 0–32)	8 (range: 0–36)	0.001
Stroke subtypes (n)				0.004
Atherothrombotic infarction	37	31	21	
Lacunar infarction	35	26	20	
Cardioembolic infarction	28	41	61	
Other types of infarction	43	43	35	
Brain hemorrhage	41	42	44	

Values are mean ± SD; GFR: glomerular filtration rate; NIHSS: National Institutes of Health Stroke scale.

failure (1 patient), cancer (3 patients) and pneumonia (5 patients). Table 2 shows a comparison of cardiovascular risk factors and stroke subtypes between the survivors and the fatal cases. The patients who died were significantly older ($p<0.001$), and had lower body mass index ($p=0.004$), lower diastolic blood pressure ($p=0.040$), lower eGFR ($p=0.021$), and higher plasma ADPN levels ($p<0.001$), higher NIHSS scores on admission ($p<0.001$). In comparison with the stroke subtypes, cardioembolic infarction contained the most fatal cases. There was no significant difference in ADPN levels between those with ischemic stroke and those with brain hemorrhage (11.2 ± 8.4 and 11.8 ± 8.6 μg/ml, respectively).

The survival rates were compared among 3 groups based on tertiles of plasma ADPN levels (Fig. 1). The highest level of ADPN was associated with poor outcome among the 3 groups in both the stroke ($p<0.001$) and all-cause mortalities patients ($p=0.001$).

The results of multivariate Cox regression analyses are shown in Tables 3a and 3b. The highest tertile of ADPN level was a significant predictor of stroke death after adjustment for age and sex (HR: 6.13, 95%CI: 1.61–23.3). Subsequently, with more adjustments for the presence of hypertension, diabetes mellitus, and hyperlipidemia in model 2, the highest tertile of ADPN remained a significant predictor of stroke mortality (HR: 6.55, 95%CI: 1.73–24.8). This association remained significant after additional adjustments for body mass index, eGFR, and NIHSS score on admission in model 3 (HR 4.69, 95% CI: 1.10–20.1). There was no significant interaction between ADPN level and variables for which distribution was different among tertiles of ADPN levels (data not shown). However, the highest tertile of ADPN level was not associated with all-cause mortality.

We analyzed for patients with ischemic stroke ($n=421$) as the same manner as analysis for all patients. The highest tertile of ADPN level was associated with stroke mortality (hazard ratio: 6.90, 95% CI: 1.34–35.5), but not with all-cause mortality (hazard ratio: 1.90, 95% confidence interval: 0.87–4.14) after adjustment for age and sex.

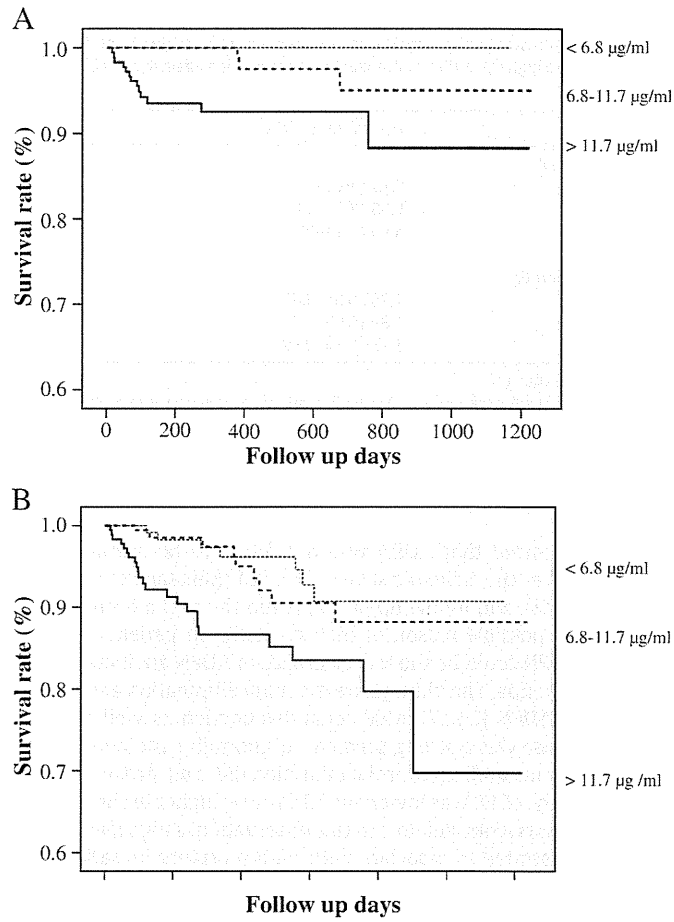

Fig. 1. Kaplan–Meier survival curves according to tertiles of plasma adiponectin concentration for (A) stroke mortality, and (B) all-cause mortality.

Table 2
Comparison of clinical parameters between survivors and fatal cases.

	Survivors (n=509)	Fatalities (n=39)	p
Age (years)	70±11	80±9	<0.001
Male (n)	341	22	0.218
Current smoker (n)	141	5	0.058
Ischemic heart disease (n)	60	2	0.295
Body mass index (kg/m^2)	23.2±3.7	21.4±3.5	0.004
Systolic blood pressure (mm Hg)	134±15	134±15	0.833
Diastolic blood pressure (mm Hg)	72±10	69±9	0.040
Total cholesterol (mg/dl)	170±37	167±36	0.603
Triglycerides (mg/dl)	113±50	102±43	0.167
LDL cholesterol (mg/dl)	108±32	107±37	0.849
HDL cholesterol (mg/dl)	42±12	41±13	0.666
Fasting plasma glucose (mg/dl)	107±35	105±27	0.800
Adiponectin (μg/ml)	10.9±8.1	16.5±10.2	<0.001
Estimated GFR, ml/min/1.73 m^2	73.1±23.5	63.9±26.8	0.021
Median NIHSS score on admission	5 (range: 0–32)	17 (range: 2–36)	<0.001
Stroke subtypes (n)			
Atherothrombotic infarction	85	4	
Lacunar infarction	81	0	
Cardioembolic infarction	111	19	
Other types of infarction			
Undetermined etiology	65	5	
Determined etiology	48	3	
Brain hemorrhage	119	8	

Values are mean±SD; LDL: low-density lipoprotein; HDL: high-density lipoprotein; GFR: glomerular filtration rate; NIHSS: National Institutes of Health Stroke scale.

4. Discussion

This is the first study demonstrating that a high level of plasma ADPN in acute stroke patients was strongly associated with a high risk of stroke death during the median follow-up period of 17 months.

Plasma ADPN levels might change by the influences of stroke severity and stroke subtypes. In this study, there was a significant positive correlation between ADPN levels and NIHSS scores on admission ($r=0.161$, $p<0.001$). Besides, ADPN knock-out mice were shown to exhibit increased cerebral infarction size [5]. Plasma ADPN levels were different in each stroke subtype in this study, consistent with a past study [16]: patients with atherothronbotic brain infarction and lacunar infarction had lower plasma ADPN levels and those with cardioembolic brain infarction had the highest.

ADPN was reported to increase energy expenditure through a direct action in the central nervous system in mice [17]. An increased level of plasma ADPN was observed in patients with heart failure with cachexia [18]. The authors suggested that the increased ADPN, which might occur in an attempt to normalize fatty acid metabolism, would cause body weight loss in patients with advanced heart failure. Recently, high levels of ADPN in patients with CVD were reported to be associated with the increased risk of mortality in both men and women [19,20]. In patients with coronary artery disease, high levels of ADPN turned out to be a risk factor for future CVD events or death [10,21,22], although another report showed no association between high levels of ADPN and CVD death [23]. Marousi S, et al. [24] reported that ADPN was significantly suppressed already by the early phases of ischemic stroke, and remained unchanged 6 months later. Prognostic implications in levels of ADPN have not been clarified. And they

Table 3a
Results of multivariate Cox regression analysis for the incidence of fatal events considering the highest tertile of adiponectin concentration against the middle plus the lowest tertiles.

	Hazard ratio (95% CI)	p
Stroke mortality		
Model 1	6.13 (1.61–23.3)	0.008
Model 2	6.55 (1.73–24.8)	0.006
Model 3	4.69 (1.10–20.1)	0.037
All-cause mortality		
Model 1	1.75 (0.88–3.48)	0.112
Model 2	1.89 (0.95–3.77)	0.070
Model 3	1.19 (0.54–2.62)	0.675

CI: confidence interval.
Model 1: adjusted for age and sex. Model 2 includes the covariates in model 1 plus the presence of hypertension, diabetes mellitus, and hyperlipidemia. Model 3 includes the covariates in model 2 plus body mass index, NIHSS score on admission, and estimated glomerular filtration rate.

recently reported that ADPN was not found to be associated with mortality after the ischemic stroke [25]. But their sample size is very small (n = 82), and follow up period is too short (6 months).

Another possible reason of high mortality in patients with high levels of ADPN could be the low clearance of ADPN attributable to the renal dysfunction. The kidneys are the main elimination apparatus for circulating ADPN [26,27]. Mild renal dysfunction as well as chronic kidney disease was a strong predictor of mortality and poor outcome in both patients with myocardial infarction [28] and stroke [29]. In the present study, eGFR was lower and ADPN was higher in the fatal cases than in the survivors. Relative to this observation, a high level of ADPN was demonstrated to associate with high mortality in patients with chronic kidney disease [11].

The present study was observational, and can only demonstrate associations. We speculate that ADPN plays a protective role in vascular injuries through exerting anti-atherosclerosis or anti-inflammatory effects; however, in high risk patients, such as CVD patients, ADPN level were raised to compensate for vascular injuries, which could result in harmful effects, notably, body weight loss and sarcopenia.

The limitations of this study are that the present investigation was conducted at a single-center with a prospective design. The sample size as well as the follow-up period might not be large enough to have a sufficient statistical power. However we demonstrated a strong association between high ADPN levels and increased risk of stroke mortality. Another limitation is that we measured total ADPN in the present study. It is known that ADPN consists of isoforms with various molecular weights (low-molecular weight; LMW, medium molecular weight; MMW, and high-molecular weight; HMW). These isoforms have different binding affinities for the ADPN receptors, and may have different bioactivities. Recently, a number of reports showed that HMW form of ADPN has more biological activity than LMW or MMW ADPN [30,31]. HMW ADPN, especially the HMW to total ADPN ratio, was significantly lower in patients with coronary artery disease than those without coronary artery disease in patients with type 2 diabetes [31]. Further analysis of ADPN isoforms with respect to cerebrovascular risk factors might clarify the contribution of ADPN to the pathogenesis of cerebrovascular disease.

Table 3b
Hazard ratio of each variable in Cox regression analysis (Model 3).

	Stroke death		All-cause death	
	Hazard ratio	p	Hazard ratio	p
Adiponectin level	4.69 (1.10–20.1)	0.037	1.19 (0.54–2.62)	0.675
Age	1.06 (0.99–1.13)	0.124	1.07 (1.03–1.11)	0.001
Sex	1.38 (0.46–4.16)	0.569	1.07 (0.53–2.16)	0.847
Hypertension	1.81 (0.39–8.32)	0.447	1.82 (0.74–4.46)	0.190
Diabetes mellitus	3.73 (1.10–12.7)	0.035	1.72 (0.78–3.80)	0.183
Hyperlipidemia	0.43 (0.11–1.67)	0.221	0.41 (0.18–0.93)	0.032
Body mass index	0.88 (0.74–1.06)	0.177	0.94 (0.84–1.05)	0.247
NIHSS score on admission	1.08 (1.01–1.16)	0.024	1.11 (1.07–1.16)	<0.001
Estimated GFR	1.01 (0.99–1.03)	0.377	1.00 (0.98–1.01)	0.537

NIHSS: National Institutes of Health Stroke scale; GFR: glomerular filtration rate.

In conclusion, a high level of ADPN in acute stroke patients was associated with an increased risk of stroke death during the 17 months of median follow up period after stroke. Further experimental and epidemiological studies are needed to elucidate possible roles of ADPN in cerebrovascular diseases.

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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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Keywords: aging; endothelial cells; focal ischemia; hematopoietic stem cell transplantation; nitric oxide

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

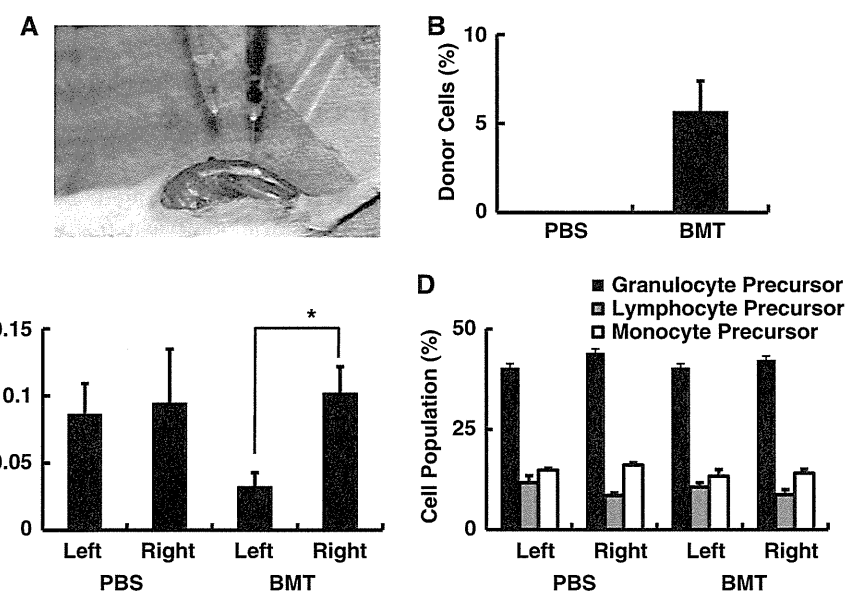

Figure 1 Transplantation of bone marrow from young rats into aged rats. (**A**) Schematic depiction of the intrabone marrow injection. (**B**) Fluorescence *in situ* hybridization (FISH) analysis of peripheral blood on day 28 after bone marrow transplantation. About 5% of circulating cells were donor 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

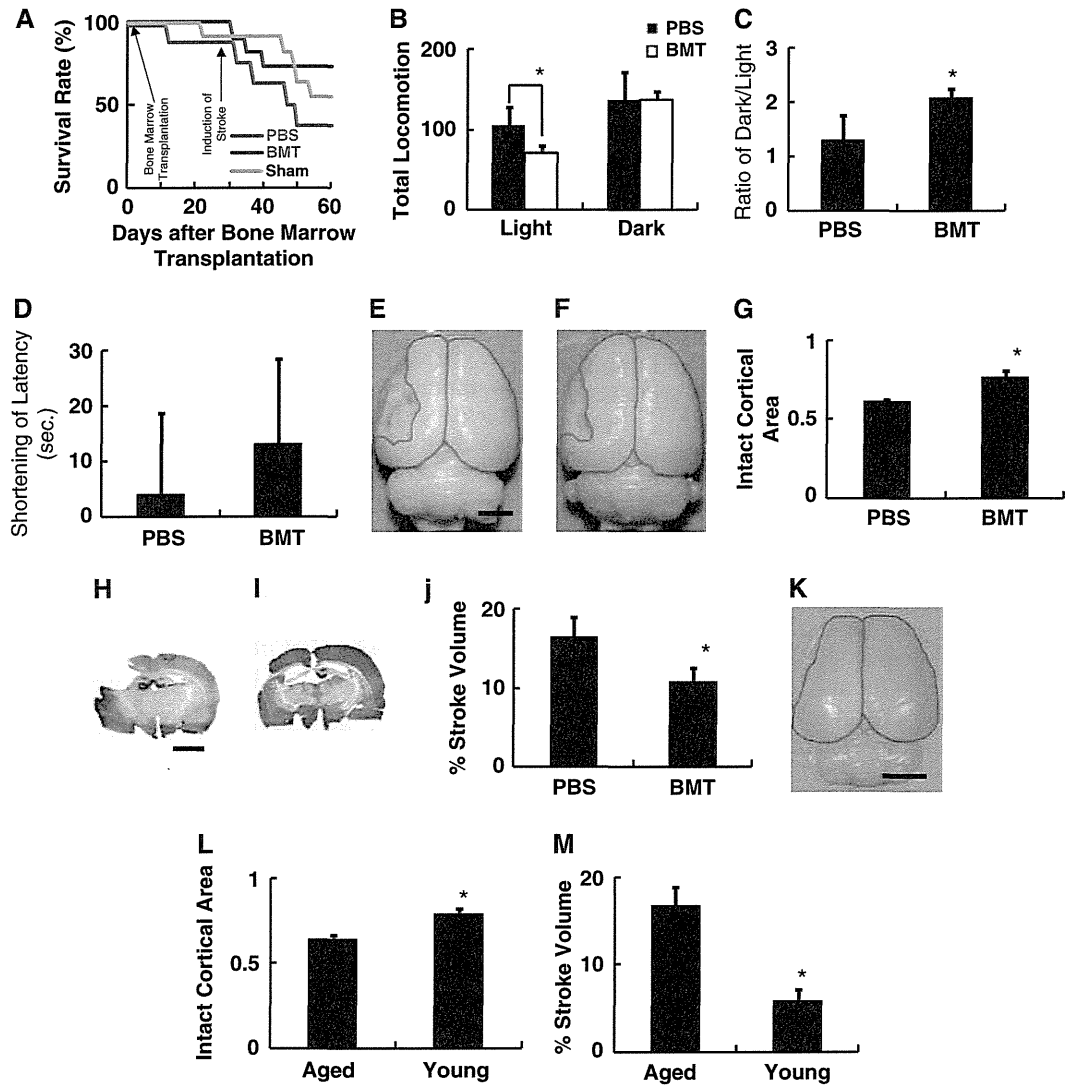

Figure 2 Transplantation of bone marrow from young rats into aged animals reduced ischemic damage in the poststroke period. (**A**) Temporal profile of animal survival for 60 days after bone marrow transplantation. (**B**) Aged animals subject to transplantation with bone marrow cells from young rats (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

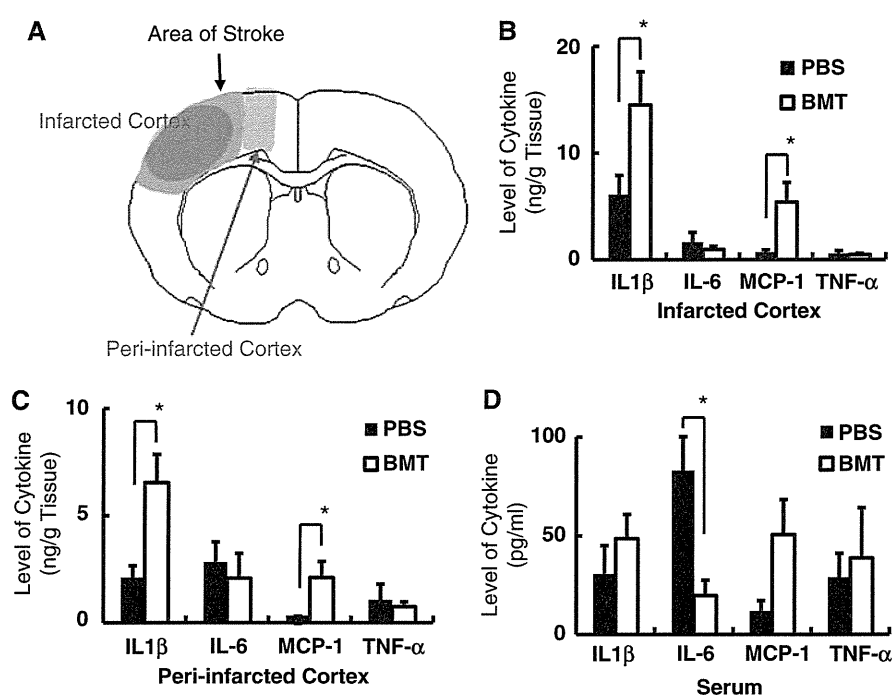

Figure 3 Profile of inflammatory cytokines in poststroke brain. (**A**) Schematic of the brain area from which tissue samples were harvested. (**B**) A significant increase in interleukin (IL)-1β and monocyte chemoattractant protein-1 (MCP-1) was observed following bone marrow transplanted (BMT) of bone 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.

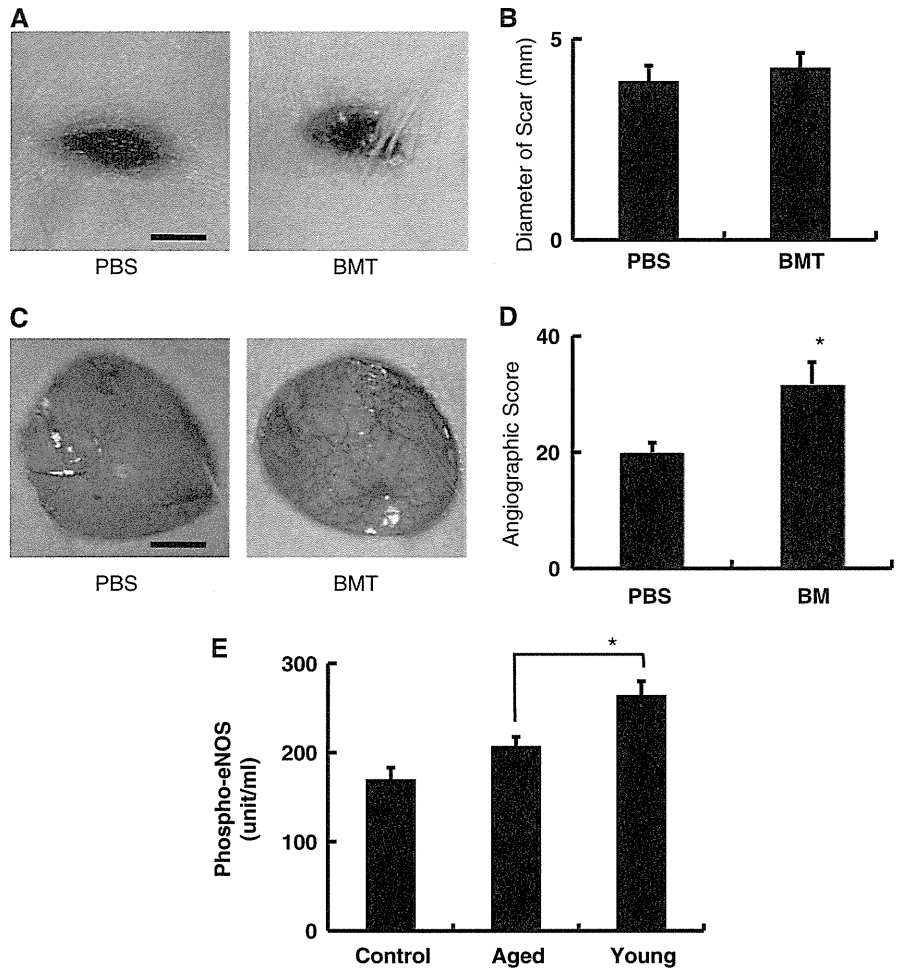

Figure 4 Endothelial cell activation: effect of bone marrow cells *in vivo* and *in vitro*. (**A**) Representative picture on day 10 after skin injury induced in aged rats treated with phosphate-buffered saline (PBS) or subject to bone marrow transplanted (BMT) with bone marrow from young rats (as described in the text). (**B**) There was no significant difference in the size of the residual wound on day 10 after skin injury comparing the two groups. (**C, D**) Representative photograph 30 seconds after skin injury. (**C**) A significant increase in the number of activated microvascular structures under the skin was observed in aged animals in the BMT group (the procedure for quantification of the angiographic score is described in the Materials and methods section) (**D**). (**E**) *In vitro* analysis revealed the enhanced activation of endothelial nitric oxide synthase (eNOS) in cultured human umbilical vein endothelial cells (HUVECs) exposed to bone marrow mononuclear cells harvested from young animals, versus those harvested from aged animals. $*P < 0.05$ versus PBS control (**D**) or aged bone marrow (**E**). $n = 5$, in each group. Scale bar: 2 mm.

monitored repair of full-thickness skin wounds made on the backs of animals using a biopsy punch. On day 10 after injury, the size of the scar was measured. No significant difference in the size of wounds was observed between animals transplanted with young bone marrow cells or PBS controls (Figures 4A and 4B). However, low power micrographs soon after injury (30 seconds) displayed many more visually apparent vascular structures in wounds from animals receiving transplanted cells from young animals compared with PBS controls (Figures 4C and 4D). As the latter occurred quickly after injury, it could be considered an acute vascular response to the skin wound (i.e., activation of microvasculature involving recruitment of additional channels or dilation of channels already subject to blood flow) rather than formation of new vascular channels, which would, presumably, require additional time.

The above observation suggested the possibility that activation of endothelial cells might occur more readily in the presence of bone marrow cells. To assess this possibility *in vitro*, HUVECs were cultured in the presence of bone marrow mononuclear cells derived from young (4 weeks old) or aged (50 weeks old) SHR-SP. As an index of endothelial activation, phosphorylation of eNOS in HUVECs was monitored. Endothelial cells cocultured with bone marrow-derived mononuclear cells from young animals displayed a higher level of phospho-eNOS, compared with HUVECs exposed to bone marrow cells from aged rats (Figure 4E).

Transplantation of Bone Marrow from Young Animals Impacts on the Cerebral Microvasculature Poststroke

To investigate the possible mechanisms underlying reduced brain damage observed poststroke in aged rats subject to transplantation with bone marrow cells from young animals, cerebrovascular density and proliferation were studied 30 days after induction of stroke. Compared with PBS-injected rats (Figure 5A), vascular density in the peristroke area of transplanted animals showed a significant increase in vascular density (Figures 5B and 5C). In contrast, there was no significant difference in the density of vasculature in the contralateral cortex comparing animals treated with PBS (Figure 5D) or bone marrow transplantation (Figures 5E and 5F). To evaluate the effect of aging on vascular density, cerebral infarction was induced in 8-week-old SHR-SP, and the density of vasculature was evaluated. The results demonstrate a significantly higher vascular density in the peristroke area (Figures 5G and 5H) and contralateral cortex (Figures 5I and 5J) in young rats compared with aged rats. These results are consistent with previous reports that cerebrovascular density decreases with aging (Hutchins *et al*, 1996; Lynch *et al*, 1999; Sonntag *et al*, 1997). To investigate proliferation of endothelium in the cerebrovasculature of the cerebral cortex, cells coexpressing Ki67 and Lectin were investigated. However, the number of cells expressing both markers was too small (mean number of double-positive cells was less than one cell per section in both group) to perform quantitative analysis and obtain meaningful data.

To evaluate the effects of bone marrow cells on endothelial cells, activation of MAP kinases was evaluated *in vitro*. The MAP kinases are serine/threonine-specific protein kinases that regulate critical cellular activities, such as mitosis, differentiation, and cell survival/apoptosis (Kant *et al*, 2006). Exposure of cultured endothelial cells to bone marrow mononuclear cells from young animals resulted in decreased levels of phospo-p38 compared with endothelial cells exposed to bone marrow from older rats (Figure 5K). As the latter is consistent with decreased activation of p38 (activation of p38 in endothelium induces apoptotic death through a mitochondrial pathway) (Mehta *et al*, 2007), it would suggest a basis for increased survival of endothelial cells in the presence of bone marrow mononuclear cells from young animals. In contrast, there were no significant differences in the phosphorylation of two other MAP kinases, ERK1/2 and JNK1/2, comparing the two groups (data not shown). These results are consistent with our *in vivo* observation that transplantation of young bone marrow into aged mice has little impact on endothelial proliferation, but enhances the density of microvasculature in the peristroke area.

To investigate the effect of transplantation of bone marrow from young mice into aged mice on microglia, sections of poststroke brain were stained with anti-Iba-1 antibody. Compared with PBS-treated rats (Figure 5L), a significant decrease in microglia was observed in the peristroke area in bone marrow transplanted rats versus nontransplanted animals (Figures 5M and 5N). In contrast, no significant difference was observed in the number of microglia in the contralateral cortex comparing PBS-treated rats and bone marrow transplanted rats (29.1 ± 1.6 and 27.2 ± 1.7/field, respectively; $P = 0.45$).

Discussion

We have demonstrated that partial rejuvenation of bone marrow from aged rats with bone marrow-derived mononuclear cells from young animals reduces damage after experimental cerebral ischemia in the SHR-SP rat strain.

Intravenous infusion of bone marrow-derived cells without pretreatment for transplantation (such as irradiation) is associated with a very low seeding efficiency of transplanted cells into host bone marrow. However, pretransplant radiation of individuals not requiring complete repopulation of bone marrow with transplanted cells (as would be true for patients with cerebral ischemia, especially during the immediate period of ischemic stress) is not likely to be clinically acceptable, due to short- and long-term complications. Thus, use of intrabone marrow administration of bone marrow-derived mononuclear cells, in addition to intravenous administration of bone marrow cells, was used for our studies. Using our protocol (i.e., no pretreatment for bone marrow transplantation, followed by intravenous and intrabone marrow administration of bone marrow cells), the level of engraftment resulted in $\approx 5\%$ chimera of transplanted versus host cells in peripheral blood. Furthermore, analysis of peripheral blood from transplanted animals showed no significant difference in mature cell populations between the two groups. Although the overall change in health status of the aged rats because of transplantation of bone marrow cells is difficult to evaluate, these results indicate that the beneficial effect of transplanting bone marrow-derived mononuclear cells from young SHR-SP into old animals, in terms of limiting brain injury following ischemia, is not simply due to optimization of the level of circulating mature cells.

Initially, we suspected that our bone marrow transplant protocol would result in a change in the cytokine response to cerebral ischemia consistent with diminished inflammation and enhanced repair. However, levels of IL-1β and MCP-1, considered deleteriously inflammatory (Chen *et al*, 2003; Holmin and Mathiesen, 2000), increased (Figure 3), and levels of IL-6, considered protective (Loddick *et al*, 1998), was not increased (Figure 3), following bone marrow transplantation. These findings indicate that beneficial effects of transplanting young