Subjects and Methods

A retrospective analysis was performed on 72 patients with carotid TIA who were admitted to our department during the interval from May 1998 to July 2005, and who underwent DWI studies within 14 days of symptom onset after the last TIA. Fifty-two patients were male. The mean (± SD) age of patients was 69 ± 10 years. Patients with isolated amaurosis fugax were excluded. MRI was performed using a Siemens Magnetom Vision 1.5-tesla MR unit. DWI scanning was performed with a single-shot, multislice spin echo and echo planar imaging sequence. DWI parameters comprised: TE = 123 ms; FOV = 23 × 23 cm; matrix = 128 × 200, and slice thickness = 4 mm. Diffusion gradients were applied in the through-plane direction with a b value of 1,100 s/mm^2. Since 1999, imaging parameters have been changed to TE = 100 ms and matrix = 98 × 128. Diffusion gradients were applied in each x, y, and z direction with b values of 1,000 s/mm^2, and trace imaging was calculated. Conventional MRI studies included T_1-weighted (TR/TE: 630/14) and T_2-weighted (TR/TE: 5,400/99) images, and fluid attenuation inversion recovery (TR/TE/TE: 9,000/105/2,400) images were obtained when required.

We assessed whether each patient had ischemic lesions and/or arterial disease compatible with symptoms by reviewing DWI films and the results of conventional cerebral angiography, MR angiography, and carotid ultrasonography. We assessed the presence or absence of cardiac embolic sources based on 12-lead ECG findings, transthoracic and/or transesophageal echocardiography and, when required, 24-hour ECG monitoring. Referring to the TOAST classification [9], all patients were classified into four groups: large-artery atherosclerosis (LA) group, cardioembolism (CE) group, small-artery occlusion (SA) group, and other etiology or undetermined etiology (UD) group. The LA group included patients with more than 50% stenosis of intracranial or extracranial large arteries or with a complicated lesion of more than 3.5 mm in the aortic arch based on findings of conventional cerebral angiography, MR angiography, carotid ultrasonography and transesophageal echocardiography. Patients in this group should not have had significant heart disease. The CE group included patients with significant heart disease that can become an embolic source, such as mechanical prosthetic valves, mitral stenosis with atrial fibrillation, atrial fibrillation, left atrial/atrial appendage thrombus, sick sinus syndrome, recent myocardial infarction within 4 weeks prior to the study, left ventricular thrombus, dilated cardiomyopathy, akinetic left ventricular segment, atrial myxoma, infective endocarditis or patent foramen ovale with peripheral thrombus but without LA. The SA group included patients who had neither significant heart disease nor LA, nor other evidence of disease. The TIA symptoms in this group should have corresponded to any of the traditional clinical lacunar syndromes and should not have been associated with cortical symptoms. The UD group included patients who could not have been classified into other groups because of the following reasons: (1) they had other causes of cerebral ischemia, such as dissection of cervical/cranial arteries, vasculitis or hypercoagulopathy, (2) they had cortical symptoms in spite of an absence of association with significant heart disease, large-artery lesions or evidence of other diseases, and (3) they had both significant heart disease and more than 50% stenosis in a large artery or aortic complicated lesions greater than 3.5 mm. This classification was made by mutual agreement by three neurologists. We also examined the duration of TIA symptoms and the time from onset of TIA to DWI in each patient. Furthermore, we reviewed the correlation between these factors and the detectability of lesions. We also studied whether patients had risk factors for atherosclerosis such as hypertension, diabetes mellitus, hyperlipidemia and smoking, and whether they had a history of cerebral infarction.

Statistical Analysis

Statistical analysis was performed using a commercially available software package (Statview, version 5, SAS Institute Inc., Cary, N.C., USA). Data were expressed as means ± SD. The level of $p < 0.05$ was determined to indicate statistical significance. We statistically compared the four groups as classified above using one-way factorial ANOVA or the Kruskal-Wallis test.

The table of baseline patient characteristics was analyzed using the Yates corrected χ^2 or Fisher test, as appropriate.

Results

Twenty-four of 72 patients (33%) had small ischemic lesions on DWI. There was no significant difference in baseline characteristics between patients with positive DWI lesions and those with negative DWI lesions (table 1). As shown in table 2, the duration of symptoms was significantly longer in patients with positive DWI lesions (4.0 ± 5.1 h) than in those with negative DWI lesions (1.4 ± 2.5 h) ($p < 0.01$). The time from TIA onset to DWI study was also significantly longer in patients with positive lesions (4.5 ± 4.1 days) than in those with negative lesions (2.0 ± 3.2 days) ($p < 0.01$). The detectability of lesions increased in correlation with the duration of TIA symptoms, as shown in figure 1. The detectability of lesions was also influenced by time from TIA onset to DWI, as follows: detectability was 14% (4/29) in the group undergoing DWI at 0–12 h after TIA, 33% (5/15) in the group undergoing DWI at 12–24 h after TIA, 43% (3/7) in the group undergoing DWI at 1–3 days after TIA, 60% (6/10) in the group undergoing DWI at 3–7 days after TIA, 57% (4/7) in the group undergoing DWI at 7–10 days after TIA, and 50% (2/4) in the group undergoing DWI at 10–14 days after TIA. Thus, the detectability of lesions was somewhat lower in patients undergoing DWI within 24 h after TIA than in those undergoing DWI more than 24 h after TIA.

Cerebral angiography was carried out on 25 patients, MR angiography on 58 patients, carotid ultrasonography on 71 patients, transthoracic echocardiography on 58 patients and transesophageal echocardiography on 60 patients. The type of etiology was classified as LA group, 25 patients; CE group, 14 patients; SA group, 13 patients, and UD group, 20 patients. The breakdown of the 20 patients

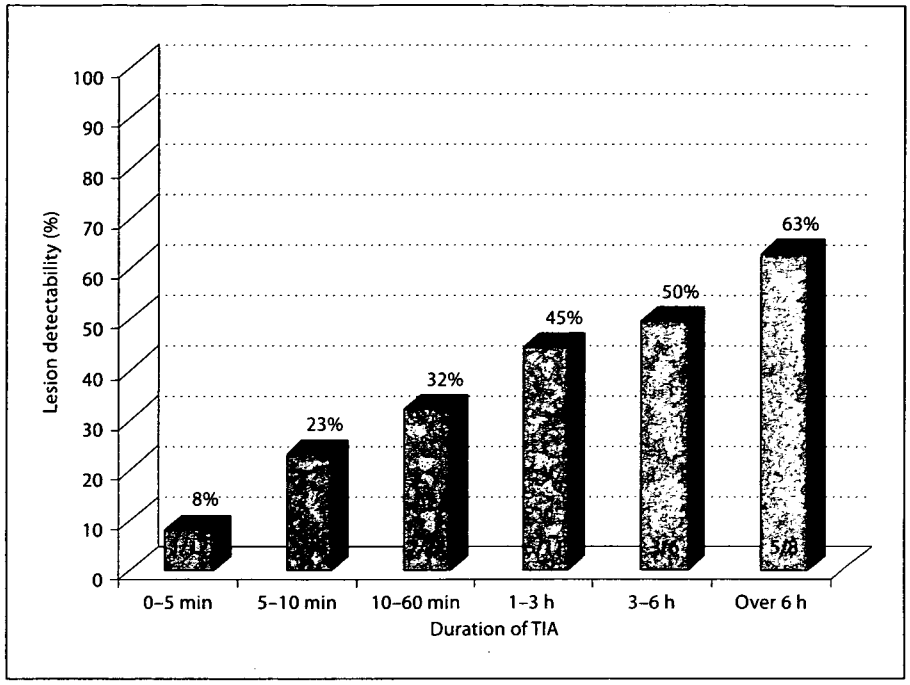

Fig. 1. The duration of TIA symptoms and DWI lesion detectability. The DWI lesion detectability increases in correlation with the duration of TIA symptoms.

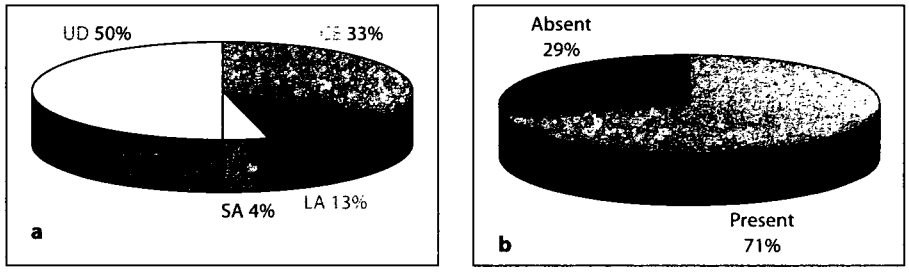

Fig. 2. Details of 24 TIA patients with DWI-positive lesions. **a** 83% of DWI-positive patients belong to the CE or UD groups. **b** 71% of DWI-positive patients have heart disease, either independently or in association with other etiologies.

in the UD group was as follows: (1) 1 patient with both antiphospholipid antibody syndrome and a significant lesion in a large artery, and 1 other patient with cervicocranial dissection and significant atherosclerotic lesions in a large artery, (2) 2 patients with cortical symptoms in association with no abnormality in the heart, large artery and other tests, and (3) 16 patients with both significant heart disease and significant stenotic lesions in large cerebral arteries and/or aorta. As shown in table 3, the detectabilities of lesions in the CE group (57%) and the UD group (60%) were significantly higher as compared with those in the LA group (12%) and the SA group (8%).

The duration of symptoms in the CE group was somewhat longer than in the other three groups, although the difference was not significant (table 3). Time from TIA onset to DWI was somewhat longer in the CE and UD groups as compared with the other two groups. However, results of one-way factorial ANOVA indicated that there was no significant difference in time from TIA to DWI studies between the groups (table 3). DWI studies were performed within 24 h after TIA in 39 patients and more than 24 h after TIA in 33 patients. Percentages of patients undergoing DWI studies within 24 h after TIA were 64% (16/25) in the LA group, 50% (7/14) in the CE group, 62% (8/13) in the SA group, and 40% (8/20) in the UD group. There were no significant differences in the frequency of early DWI studies between the four groups (Kruskal-Wallis test). The CE and UD groups had higher lesion detectability irrespective of time from TIA to DWI studies.

A total of 14 patients had multiple lesions on DWI. The frequency of multiple lesions in each group was as fol-

Table 1. Baseline patient characteristics

	DWI positive (n = 24)	DWI negative (n = 48)
Age, years	68 ± 10	69 ± 10
Male gender	18 (75)	34 (71)
Hypertension	15 (63)	34 (71)
Diabetes mellitus	4 (17)	10 (21)
Hypercholesterolemia	9 (38)	21 (44)
Smoking	13 (54)	31 (65)
History of stroke	1 (4)	8 (17)

Figures in parentheses indicate percentages.

Table 2. Duration of symptoms and time to MRI studies in DWI-positive and DWI-negative patients

	DWI positive	DWI negative
Duration of symptoms, h	4.0 ± 5.1*	1.4 ± 2.5
Time from TIA to MRI, days	4.5 ± 4.1*	2.0 ± 3.2

* $p < 0.01$: significantly longer as compared with the diffusion-negative patients.

Table 3. Comparison of four TIA groups

	LA (n = 25)	CE (n = 14)	SA (n = 13)	UD (n = 20)
Positive DWI lesions	3 (12)	8 (57)[1]	1 (8)	12 (60)[1]
Duration of symptoms, h	1.5 ± 3.0	3.8 ± 5.2	1.7 ± 2.8	2.5 ± 3.9
Time from TIA to DWI, days	1.8 ± 2.6	3.6 ± 4.1	2.6 ± 4.5	3.6 ± 4.1

Figures in parentheses indicate percentages.
[1] The frequency of DWI-positive lesions is significantly higher in the CE and the UD groups as compared with the LA and SA groups ($p < 0.05$).

Table 4. The type of heart diseases and lesion detectability in the CE group

Type of heart diseases	DWI positive	DWI negative
Atrial fibrillation	5	2
Mechanical prosthetic valve	1	1
Akinetic left ventricular segment	2	0
Sick sinus syndrome	0	1
Patent foramen ovale with peripheral thrombus	0	2

lows: in the LA group, 2 of 3 patients with positive lesions (67%) had multiple lesions; the number of lesions was 3 and 5, respectively. In the CE group, 3 of 8 patients (38%) had multiple lesions; the number of lesions was 3 in 2 cases and 4 in the other. In the SA group, 1 positive patient had only a single lesion (0%). In the UD group, 9 of 12 patients (75%) had multiple lesions; the number of lesions was 2 in 5 cases, 3 in 3 cases and 6 in the remainder. Thus, no remarkable relationship was observed between the number of lesions and the etiology.

Of all 24 patients with ischemic lesions on DWI, 8 patients (33%) belonged to the CE group, 12 patients (50%) to the UD group, 3 patients (13%) to the LA group, and 1 patient (4%) to the SA group (fig. 2). Of the 12 patients with positive DWI lesions who belonged to the UD group, 9 had significant heart disease in association with significant large-artery lesions. Thus, of all 24 patients with ischemic lesions on DWI, 17 patients (71%) had significant heart disease either independently or concomitantly with large-artery lesions (fig. 2). In the CE group, 5 of 7 patients with atrial fibrillation had positive DWI lesions (table 4).

Discussion

In recent years, quite a few studies have reported the presence of ischemic lesions on DWI following TIA. In these previous studies, the detectability of TIA lesions on DWI ranged from 20 to 70% [8, 10–16]. Detectability in the present study was 33%, showing a somewhat lower value as compared with previous studies. This may be partly related to the difference in the timing of DWI examinations between the present study and the previous studies. In the present study, approximately 54% of patients underwent DWI within 24 h after TIA onset; the detectability in these patients was low, at 21%. On the other hand, in most previous studies the majority of TIA patients underwent DWI examinations more than 24 h after TIA onset. When we calculated only the detectability of lesions in our patients undergoing DWI more than 24 h after TIA onset, the detectability increased to 48%, showing similar values to those reported in previous studies. The above-mentioned reasoning can also be inferred from the study of Rovira et al. [10]. In their study,

only 9% of patients underwent DWI within 48 h after TIA, and the detectability of lesions in the entire group showed a high value, reaching 67%. In a transient ischemia experiment using rats, the value of the average apparent diffusion coefficient decreased significantly during the ischemic period and then normalized at 60–90 min after ischemia, followed again subsequently by a significant reduction more than 12 h after ischemia [17]. As confirmed in the above experiment, the detectability of lesions on DWI may decrease for a while after a short period of transient cerebral ischemia, and may increase thereafter, although the mechanisms remain unclear. Kidwell et al. [8] first pointed out that the detectability of lesions on DWI in patients with TIA increases in correlation with the duration of symptoms. Since then, similar results have been reported by several authors. In the study by Rovira et al. [10], the detectability of lesions in TIA patients with symptoms lasting less than 6 h was 59%, whereas the value was 100% in patients with symptoms lasting more than 6 h. Crisostomo et al. [11] reported that the detectability of lesions in patients with symptoms lasting more than 1 h was significantly higher as compared with patients with symptoms lasting less than 1 h. Inatomi et al. [12] also reported that the detectability of lesions was significantly higher in TIA patients with symptoms lasting more than 30 min than in those with symptoms lasting less than 30 min. In our study, the detectability of lesions also tended to increase according to the increase in TIA duration.

Previously, few workers performed detailed investigations on the relationship between the detectability of DWI lesions and the etiology of TIA. Rovira et al. [10] reported that the detectability of DWI lesions was higher in TIA patients with large-artery lesions than in those with cardiac lesions. However, the report lacks credibility, since the number of patients in their study was small; only 4 patients had cardiac lesions, whereas 19 patients had large-artery lesions. Nakamura et al. [16] reported higher detectability of DWI lesions in TIA patients with atrial fibrillation as compared with those without atrial fibrillation. However, they focused only on atrial fibrillation and did not clarify DWI detectability in TIA patients without atrial fibrillation who had other types of heart diseases. In the present study, the presence or absence of large-artery lesions and/or cardiac disease was surveyed in a retrospective manner reviewing the results of conventional cerebral angiography, MR angiography, carotid ultrasonography, 12-lead ECG, transthoracic or transesophageal echocardiography, and 24-hour ECG monitoring. The patients were then classified into four groups according to the etiology of TIA, such as LA, CE, SA and UD groups. The results indicated that the detectability of lesions in the CE group and the UD group was higher than that in the other groups. Time from TIA to DWI studies was almost the same in the four groups. Lesion detectability was higher in the CE and UD groups than in the other groups, even when the comparison among the subgroups undergoing DWI studies had been made within 24 h after TIA onset. Therefore, the higher detectability in the CE and UD groups is unrelated to time from TIA to DWI studies. The mean duration of TIA symptoms in the CE group was more than 3 h, which was the longest of all the groups. In general, cardioembolic stroke produces severer symptoms than artery-to-artery embolic stroke. This may be attributable to the fact that emboli originating in the heart tend to occlude larger blood vessels for longer durations as compared with artery-to-artery emboli. This is probably also true in cases of TIA. Microemboli originating in the heart likely occlude larger blood vessels for longer durations as compared with artery-to-artery microemboli. Accordingly, ischemic duration may be longer in TIA patients with heart disease than in those with other types of etiology, and ischemic lesions may be larger in TIA patients with heart disease than in those with other types of etiology. Probably for such reasons, ischemic lesions in cardioembolic TIA may be more readily found on DWI than those in other types of TIA. In the present study, 16 of 20 patients in the UD group had heart disease, and 9 had ischemic lesions on DWI. In these 9 patients, TIA was most likely caused by a cardioembolic mechanism rather than another type of etiology. Johnston et al. [18] conducted a follow-up study in 1,707 patients with TIA for 90 days. In their study, 10.5% of patients developed cardioembolic stroke during the follow-up period, and approximately half of them had stroke within 48 h after TIA. Thus, cardioembolic stroke may occur soon after TIA at a considerably high frequency. A DWI study is considered useful to evaluate etiological mechanisms of TIA. If ischemic lesions are detected on DWI, the presence of heart disease should be suspected, and appropriate medication should be considered to prevent cardioembolic stroke.

Acknowledgement

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Brief Communication

Increase in circulating CD34-positive cells in patients with angiographic evidence of moyamoya-like vessels

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Increasing evidence points to a role for circulating endothelial progenitor cells, including populations of CD34-positive (CD34$^+$) cells, in maintenance of cerebral blood flow. In this study, we investigated the link between the level of circulating CD34$^+$ cells and neovascularization at ischemic brain. Compared with control subjects, a remarkable increase of circulating CD34$^+$ cells was observed in patients with angiographic moyamoya vessels, although no significant change was observed in patients with major cerebral artery occlusion (or severe stenosis) but without moyamoya vessels. Our results suggest that the increased level of CD34$^+$ cells associated with ischemic stress is correlated with neovascularization at human ischemic brain.
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Keywords: antigens; CD34; moyamoya vessel; neovascularization

Introduction

Increasing evidence points to a role for bone marrow-derived immature cells, such as endothelial progenitor cells, in maintenance of vascular homeostasis and repair. CD34-positive (CD34$^+$) cells comprise a population enriched for endothelial progenitor cells whose contribution to neovasculature includes both direct participation in forming the neovessel and regulatory roles as sources of growth/angiogenesis factors (Majka *et al,* 2001). Previously, we have shown accelerated neovascularization after administration of CD34$^+$ cells in an experimental model of stroke (Taguchi *et al,* 2004*b*) and induced by autologous bone marrow mononuclear cells (rich cell fraction of CD34$^+$ cells) transplanted locally into patients with limb ischemia (Taguchi *et al,* 2003). In addition, we have observed a positive correlation between the level of circulating CD34$^+$ cells and regional blood flow (Taguchi *et al,* 2004*a*), and cognitive function (Taguchi *et al,* 2007) in patients with chronic cerebral ischemia.

In this study, we have evaluated the level of circulating CD34$^+$ cells in patients with unusually accelerated neovascularization induced by progressive occlusion (or severe stenosis) of the supraclinoid portion of the internal carotid artery, the proximal region of the anterior, and/or middle cerebral artery characterized angiographically by the presence of moyamoya-like vessels (Natori *et al,* 1997) that supply ischemic brain as collaterals. We have investigated the hypothesis that circulating bone marrow-derived immature cells might be associated with neovascularization at ischemic sites in the human brain.

Patients and methods

The institutional review board of the National Cardiovascular Center approved this study. All subjects provided

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informed consent. A total of 50 individuals, including 24 patients with occlusion or severe stenosis (>90%) at the C1 portion of the internal carotid artery or the M1 portion of the middle cerebral artery, and 26 age-matched healthy volunteers with cardiovascular risk factors, but without history of vascular disease, were enrolled. The diagnosis of cerebral artery occlusion or stenosis was made angiographically and four patients were found to have classical angiographic evidence of moyamoya-like vessels, including one with right C1 occlusion, one with right M1 occlusion, and two with bilateral C1 severe stenosis. All patients with cerebral artery occlusion or stenosis had a history of cerebral infarction. Individuals excluded from the study included patients who experienced a vascular event within 30 days of measurements, premenopausal women, and those with evidence of infection and/or malignant disease. The number of circulating $CD34^+$ cells was quantified as described (Taguchi *et al*, 2007). In brief, blood samples (200 μl) were incubated with phycoerythrin-labeled anti-CD34 antibody, fluorescein isothiocyanate-labeled anti-CD45 antibody, 7-aminoactinomycin-D (7-AAD), and internal control (all of these reagents are in the Stem-Kit; BeckmanCoulter, Marseille, France). After incubation, samples were centrifuged, and supernatant was removed to obtain concentrated cell suspensions. 7-Aminoactinomycin-D-positive dead cells and CD45-negative cells were excluded, and the number of cells forming clusters characteristic of $CD34^+$ cells (i.e., low side scatter and low-to-intermediate CD45 staining) was counted. The absolute number of $CD34^+$ cells was calculated using the internal control. Mean cell number of duplicate measurements was used for quantitative analysis. Statistical comparisons among groups were determined using analysis of variance or χ^2 test. Individual comparisons were performed using a two-tailed unpaired Students' *t*-test or Mann–Whitney's *U*-test. Mean ± s.e. is shown.

Results

Enrolled individuals were divided into three groups: control subjects, patients with cerebral occlusion or severe stenosis, but without the presence of vessels with angiographic characteristics of moyamoya disease, and patients with angiographic evidence of moyamoya-like vessels. Baseline characteristics of the groups are shown in Table 1. The modified Rankin scale evaluation of patients with and without moyamoya-like vessels was 0.5 ± 0.5 and 1.3 ± 0.2, respectively ($P = 0.15$). Comparing these groups, there was a significant difference in the ratio of gender and treatment with aspirin between groups. However, no significant difference was observed in the number of circulating $CD34^+$ cells in control group between genders (male, $n = 13$, $CD34^+$ cells $= 0.93 \pm 0.10/\mu L$; female, $n = 13$, $CD34^+$ cells $= 0.85 \pm 0.11/\mu L$: $P = 0.59$) and treatment with aspirin (aspirin (+), $n = 6$, $CD34^+$ cells $= 0.76 \pm 0.12/\mu L$; aspirin (−), $n = 20$, $CD34^+$ cells $= 0.93 \pm 0.09/\mu L$: $P = 0.26$), indicating mild and nonsignificant effects of gender and treatment with aspirin on the level of circulating $CD34^+$ cells. In univariate analysis of control subjects, each cerebrovascular risk factor and treatment with statins showed no significant difference in the number of circulating $CD34^+$ cells (data not shown).

A representative angiogram showing characteristics of moyamoya-like vessels is shown in Figures 1A and 1B. Angiographic moyamoya-like vessels were observed around the M1 portion of an occluded middle cerebral artery. Compared with a normal subject (Figure 1C) and patients without angiographic evidence of moyamoya-like vessels (Figure 1D), a remarkable increase in levels of

Table 1 Baseline characteristics

	Total	*Control*	*Major artery occlusion/stenosis*		*P-value for trend*
			Moyamoya (−)	*Moyamoya (+)*	
N	50	26	20	4	
Age, years	60.8 ± 1.1	60.5 ± 1.9	61.5 ± 1.0	59.3 ± 5.9	0.85
Male, *n* (%)	33 (66)	13 (50)	18 (90)	2 (50)	0.01
Risk factor, n (%)					
Hypertension	35 (70)	16 (62)	15 (75)	4 (100)	0.24
Hyperlipidemia	26 (52)	14 (54)	10 (50)	2 (50)	0.96
Diabetes mellitus	11 (22)	7 (27)	4 (20)	0 (0)	0.46
Smoking	15 (30)	7 (27)	8 (40)	0 (0)	0.25
Treatment, n (%)					
Ca channel blockers	20 (40)	10 (38)	8 (40)	2 (50)	0.91
β-Blockers	5 (10)	3 (11)	1 (5)	1 (25)	0.44
ACE inhibitor	7 (14)	4 (15)	2 (10)	1 (25)	0.70
ARB	12 (24)	5 (19)	5 (25)	2 (50)	0.40
Diuretics	4 (8)	2 (7)	1 (5)	1 (25)	0.40
Statin therapy	14 (28)	9 (34)	4 (20)	1 (25)	0.54
Aspirin	19 (38)	6 (23)	10 (50)	3 (75)	0.05
Ticlopidine	12 (24)	3 (11)	8 (40)	1 (25)	0.08

Abbreviations: ACE, angiotensin-converting enzyme; ARB, angiotensin 2 receptor blocker.

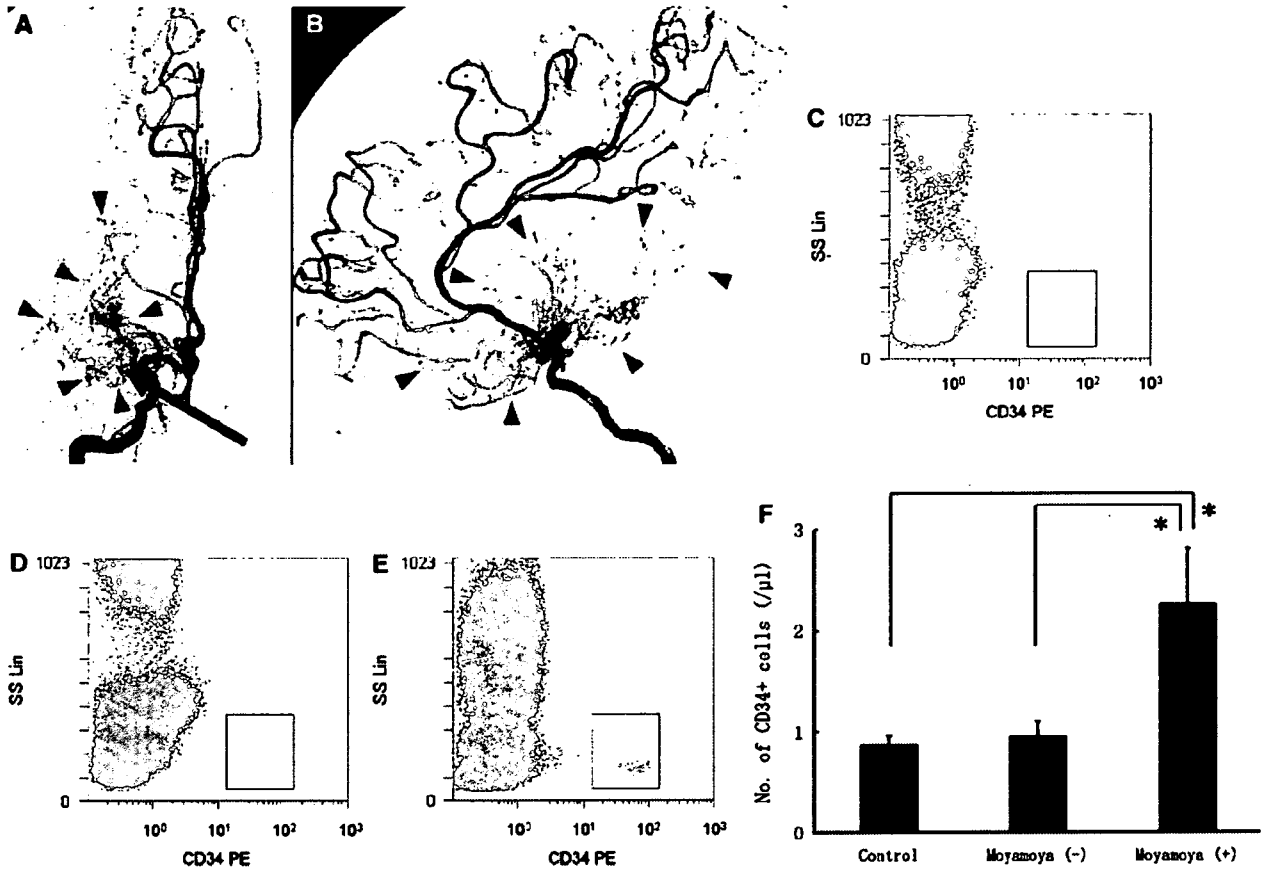

Figure 1 Increased levels of circulating CD34+ cells in patients with angiographic evidence of moyamoya-like vessels. (**A, B**) Representative angiogram from a patient with moyamoya-like vessels. Unusually accelerated neovascularization (based on angiographic features of moyamoya-like vessels, arrowheads) was observed around an occlusive M1 lesion (arrow). Anterior–posterior view (**A**) and lateral view (**B**) of the right internal carotid artery showed angiographically. (**C–E**) After exclusion of 7-aminoactinomycin-D (7-AAD)-positive dead cells and CD45-negative cells (nonleukocytes), CD34+ cells cluster at low side scatter. Representative fluorescence-activated cell sorting analyses from a control subject (**C**), a patient without moyamoya-like vessels (**D**), and a patient with moyamoya-like vessels (**E**) are shown. (**F**) A more than two-fold increase in circulating CD34+ cells was observed in patients with moyamoya-like vessels, compared with control subjects and patients without moyamoya-like vessels (*$P < 0.001$). SS Lin: side-scatter linear scale.

peripheral CD34+ cells was observed in patients with moyamoya-like vessels (Figure 1E) based on fluorescence-activated cell sorting. To confirm this impression, levels of circulating CD34+ cells were quantified (control, CD34+ cells = $0.89 \pm 0.07/\mu L$; moyamoya (−), CD34+ cells = $0.98 \pm 0.13/\mu L$; moyamoya (+), CD34+ cells = $2.28 \pm 0.53/\mu L$) and found to be significantly increased in patients with moyamoya-like vessels more than two-fold higher than in controls (Figure 1F, $P < 0.001$).

Discussion

In this study, we have found that a feature of unusually accelerated neovascularization, evidence of moyamoya-like vessels in the immediate locale of an occluded major cerebral artery, can be correlated with a robust increase in the level of circulating CD34+ cells. The latter was determined using a newly developed method that enables quantification of few CD34+ cells in peripheral blood in a highly reproducible manner.

After acute cerebral ischemia, mobilization of CD34+ cells from bone marrow has been shown in stroke patients (Taguchi *et al*, 2004*a*). Furthermore, transplantation of CD34+ cells (Taguchi *et al*, 2004*b*) and bone marrow cells (Borlongan *et al*, 2004*a*, *b*) has been shown to restore cerebral blood flow in experimental models of stroke. In chronic ischemia, transplantation of CD34+ cells has also been shown to accelerate neovascularization, including formation of collateral vessels, in patients with chronic ischemic heart disease (Boyle *et al*, 2006) and limb ischemia (Kudo *et al*, 2003). In addition, there is a report regarding the correlation between inadequate coronary collateral development and reduced numbers of circulating endothelial progenitor cells in

patients with myocardial ischemia (Lambiase *et al*, 2004). In this study, we show, for the first time, a correlation between neovascularization of the cerebral arterial circulation and increased levels of circulating $CD34^+$ cells. Our results support the hypothesis that circulating $CD34^+$ cells potentially contribute to neovascularization at sites of ischemic brain injury.

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Conflict of interest

The authors state no conflict of interest.

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Circulating CD34-Positive Cell Number Is Associated With Brain Natriuretic Peptide Level in Type 2 Diabetic Patients

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Patients with type 2 diabetes often suffer from asymptomatic left ventricular (LV) injury, including increased LV mass, without apparent myocardial ischemia. The mechanisms underlying diabetic LV injury remain unclear; however, it has been suggested that endothelial dysfunction plays a role. Accumulating evidence indicates that bone marrow–derived endothelial progenitor cells (EPCs) contribute to neovascularization of ischemic tissue and endothelialization of denuded endothelium. Recent studies have shown that circulating bone marrow–derived immature cells, including $CD34^+$ cells, contribute to the maintenance of the vasculature, both as a pool of EPCs and as the source of growth/angiogenesis factors (1). We hypothesized that circulating $CD34^+$ cells might be associated with LV dysfunction in patients with type 2 diabetes. Therefore, we studied the correlation between circulating $CD34^+$ cell levels and plasma brain natriuretic peptide (BNP) levels, an LV dysfunction marker, in type 2 diabetic patients.

RESEARCH DESIGN AND METHODS

The institutional review board of the National Cardiovascular Center approved this study, and all subjects provided informed consent. We examined 26 patients with type 2 diabetes (12 men and 14 women, duration of diabetes 16.1 ± 10.7 years) who were over 60 years of age (70.5 ± 6.4 years). Statin was given to nine subjects. ACE inhibitor or angiotensin receptor blocker was given to nine subjects, and thiazolidinedione was given to two subjects. Subjects were excluded from the study if they had known cardiovascular disease or chronic renal failure (defined as serum creatinine ≥180 μmol/l). No study subject showed hypokinesis by echocardiography or electrocardiogram change, indicating myocardial ischemia. Systolic (SBP) and diastolic (DBP) blood pressure and anthropometric parameters were determined. Blood samples were taken after 12-h fasting to measure circulating $CD34^+$ cells, plasma BNP, fasting plasma glucose (FPG), and A1C. Circulating $CD34^+$ cells were quantified by flow cytometry according to the manufacturer's protocol (ProCOUNT; Becton Dickinson Biosciences) as previously reported (2). BNP was quantified by enzyme immunoassay (Tohso, Tokyo, Japan). We further examined LV fractional shortening (LVFS), LV mass index (LVMI) (3), and peak flow velocity of the early filling wave (E), the late filling wave (A), and the E/A-wave ratio (E/A) by echocardiography. All echocardiograms were performed by several expert physicians who were blinded to $CD34^+$ cell level.

All statistical analyses were performed using JMP version 5.1.1 software (SAS Institute). Data are expressed as means ± SD. Comparisons of number of $CD34^+$ cells by sex were made using the two-tailed unpaired *t* test. Correlations between number of $CD34^+$ cells and clinical parameters were assessed by univariate liner regression analysis and multiple regression analysis. LVMI and plasma BNP concentrations were analyzed after logarithmic transformation.

RESULTS

FPG levels, A1C levels, and BMIs in the study subjects were measured to be 9.5 ± 2.6 mmol/l, 9.2 ± 1.8%, and 26.4 ± 4.3 kg/m^2, respectively. A total of 88% of the patients had hypertension (SBP 142 ± 18 mmHg, DBP 75.7 ± 13.5 mmHg). Plasma BNP levels were measured to be 95 ± 319 pg/ml. Although it has been reported that the level of BNP ≥100 pg/ml has a sensitivity of 90% of diagnosing congestive heart failure (CHF) in patients with CHF symptoms (4), none of the subjects in this study, including subjects with ≥100 pg/ml of BNP, showed symptoms of CHF. The level of circulating $CD34^+$ cells was measured to be 0.76 ± 0.39 cells/μl, and there was no significant difference between sexes. The range of LVMI was 73.3–340.2, and 11 subjects applied to the definition of LV hypertrophy (LVMI ≤131 in men and ≤100 in women) (3).

Plasma BNP levels had a significant inverse correlation with the number of circulating $CD34^+$ cells (Fig. 1*A*), whereas FPG, A1C, BMI, SBP, DBP, and age showed no significant correlations. There was a significant correlation between the number of circulating $CD34^+$ cells and LVMI by echocardiography (Fig. 1*B*). LVFS and E/A were not associated with circulating $CD34^+$ cell numbers (LVFS $r = -0.07$, $P = 0.72$; E/A $r = -0.11$, $P = 0.59$). There was also a significant correlation between BNP levels and LVMI ($r = 0.59$, $P = 0.001$).

In multiple regression analysis, the

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Abbreviations: BNP, brain natriuretic peptide; CHF, congestive heart failure; DBP, diastolic blood pressure; EPC, endothelial progenitor cell; FPG, fasting plasma glucose; LV, left ventricular; LVFS, LV fractional shortening; LVMI, LV mass index; SBP, systolic blood pressure.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

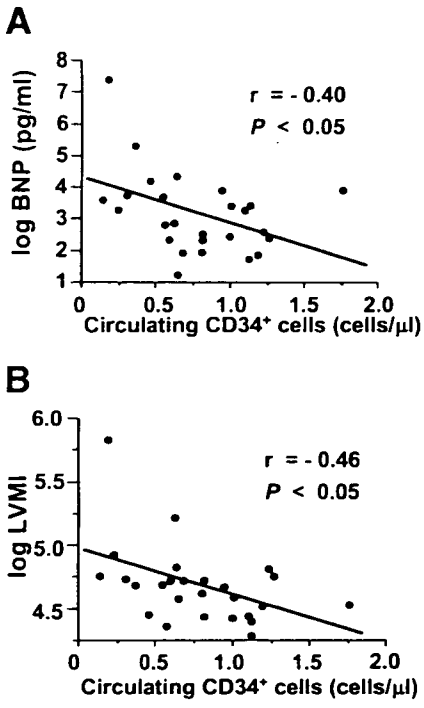

Figure 1—*Correlation between CD34$^+$ cell numbers and plasma BNP levels (A) and correlation between CD34$^+$ cell numbers and LVMI (B) in type 2 diabetic patients* (n = 26).

level of CD34$^+$ cells was an independent correlate of both BNP ($\beta = -1.64$, $P = 0.017$) and LVMI ($\beta = -0.337$, $P = 0.031$) in the model including age, A1C, SBP, BMI, and medication (ACE inhibitor/angiotensin receptor blocker, statin, and thiazolidinedione).

CONCLUSIONS — In this study, circulating CD34$^+$ cell number was found to significantly correlate with plasma BNP level, a marker of LV dysfunction. To the best of our knowledge, this is the first report that circulating bone marrow–derived cells are associated with diabetic LV abnormality. Circulating CD34$^+$ cell numbers also significantly correlated with LVMI, whereas they did not correlate with LVFS (an LV systolic function marker) or E/A (an LV diastolic function marker). LV hypertrophy is a well-known predictor of cardiovascular events independent of coronary artery disease. The Framingham Heart Study identified an association between diabetes and increased LV wall thickness and mass (5). Although the precise mechanisms underlying the association between diabetes and LV hypertrophy remain unknown, our results suggest that reduced circulating CD34$^+$ cell numbers may be involved in the progression of LV hypertrophy in diabetic patients. However, further investigations are necessary to demonstrate this hypothesis.

We measured the level of CD34$^+$ cells in this study but not the levels of circulating CD34$^+$/kinase insert domain receptor (KDR)$^+$ cells that are regarded as EPCs. Circulating CD34$^+$ cell levels are associated with ischemic stroke (6), and administration of CD34$^+$ cells ameliorates cerebral ischemia in mice (7). This indicates that CD34$^+$ cells may be involved in cardiovascular disease. Indeed, another recent report indicated that levels of circulating CD34$^+$ cells are more strongly correlated with cardiovascular risk than levels of EPCs (8). Therefore, our results suggest that measurement of CD34$^+$ cells may provide an indicator for diabetic LV hypertrophy.

Our study had several limitations. First, the study was performed only by cross-sectional analysis; therefore, a prospective study is needed to clarify whether circulating CD34$^+$ cell numbers predict LV injury in diabetic patients. Second, although systemic blood pressure did not significantly associate with CD34$^+$ cell numbers, further investigation of normotensive diabetic patients is needed to exclude the possible effects of hypertension on circulating CD34$^+$ cell numbers, as most of the subjects in this study were hypertensive. Despite this caveat, these results may be of practical use in elderly patients with type 2 diabetes, as hypertension is a very common comorbid condition in this population.

In conclusion, reduced circulating CD34$^+$ cell numbers are significantly associated with plasma BNP concentration and LVMI in elderly patients with type 2 diabetes. These results suggest that decreased circulating CD34$^+$ cells may be involved in LV hypertrophy and that measurement of circulating CD34$^+$ cell numbers may be useful for the identification of diabetic patients at high risk of LV injury.

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Brief Communication

Circulating CD34-positive cells provide a marker of vascular risk associated with cognitive impairment

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Maintenance of uninterrupted cerebral circulation is critical for neural homeostasis. The level of circulating CD34-positive (CD34$^+$) cells has been suggested as an index of cerebrovascular health, although its relationship with cognitive function has not yet been defined. In a group of individuals with cognitive impairment, the level of circulating CD34$^+$ cells was quantified and correlated with clinical diagnoses. Compared with normal subjects, a significant decrease in circulating CD34$^+$ cells was observed in patients with vascular-type cognitive impairment, although no significant change was observed in patients with Alzheimer's-type cognitive impairment who had no evidence of cerebral ischemia. The level of cognitive impairment was inversely correlated with numbers of circulating CD34$^+$ cells in patients with vascular-type cognitive impairment, but not Alzheimer's type. We propose that the level of circulating CD34$^+$ cells provides a marker of vascular risk associated with cognitive impairment, and that differences in the pathobiology of Alzheimer's- and vascular-type cognitive impairment may be mirrored in levels of circulating CD34$^+$ cells in these patient populations.
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Keywords: antigens; CD34; cerebral circulation; cognitive impairment

Introduction

Maintaining integrity of the cerebral circulation has a critical role in neural homeostasis. Although analysis of risk factors for cerebrovascular disease has certainly provided insights into mechanisms of vascular disease, it is still difficult to predict accurately the contribution of vascular dysfunction in the long-term outcome of acute vascular insufficiency or in chronic neurodegenerative disorders. For example, in Alzheimer's disease (Casserly and Topol, 2004; Vagnucci and Li, 2003), assessment of a possible vascular component in the pathogenesis of neuronal degeneration is often ambiguous during a patient's lifetime.

Repair of the cerebral microcirculation has traditionally been assigned to ongoing replacement of damaged cerebral endothelium from outgrowth of preexisting vasculature. However, recent studies have identified circulating bone marrow-derived immature cells, including CD34-positive (CD34$^+$) cells, as contributors in maintenance of the vasculature; they have the potential to serve as a pool of endothelial progenitor cells (Asahara *et al*, 1997) and as a source of growth/angiogenesis factors (Majka *et al*, 2001). In a previous study, we have shown that circulating CD34$^+$ cells provide an index of cerebrovascular function (Taguchi *et al*, 2004*a*). We have also found that in a model of experimental cerebral ischemia, intravenous administration of CD34$^+$ cells improved neurologic function, at least in part, by restoring cerebral microcirculation in the ischemic area (Taguchi *et al*, 2004*b*).

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These results lead us to propose that circulating immature vascular progenitor cells contribute to neural homeostasis, at least in part, through their role in maintaining cerebral microvascular function. Using a recently developed method that allows precise measurement of the $CD34^+$ cell population in peripheral blood (Kikuchi-Taura *et al*, 2006), we have evaluated the level of circulating $CD34^+$ cells in patients with impaired neurologic function of diverse etiologies. Our goal has been to determine if there is relationship between levels of $CD34^+$ cells, impaired neural function, and vascular integrity.

Materials and methods

This study was approved by Institutional Review Boards of the respective institutions (National Cardiovascular Center, Hyogo College of Medicine, Hoshigaoka Koseinenkin Hospital, and Osaka Minami National Medical Center). All subjects provided informed consent. Individuals with Mini Mental State Examination Score (MMSE) <24 and Clinical Dementia Rating (CDR) ≥ 0.5 were enrolled in this study and defined as having impaired cognitive function. In the view of history, evaluation of symptoms, and results of brain imaging studies (magnetic resonance imaging and single photon-computed tomography), patients with cognitive impairment were divided into two groups by neurologists blinded to the experimental protocol: vascular-type cognitive impairment or Alzheimer's-type cognitive impairment, according to the criteria of *Diagnostic and Statistical Manual of Mental Disorders* (4th ed, DSM-4) (American Psychiatric Association, 1994). To exclude the contribution of vascular element in patients with Alzheimer's-type cognitive impairment, patients' coexistent Alzheimer's-type cognitive impairment and cerebral infarction, observed by magnetic resonance imaging, were excluded from this study. In addition, patients with cognitive impairment diagnosed as neither of the Alzheimer's type nor vascular type were excluded. A total of 95 individuals, including 32 age-matched control subjects with no history of vascular disease, no neuronal deficiency, and no cognitive impairment, were enrolled. In addition, individuals excluded from the study included: premenopausal women, patients who experienced a vascular event within 30 days of measurements, history of cerebral hemorrhage, and evidence of infection or malignant disease. Using a modification of the International Society of Hematotherapy and Graft Engineering (ISHAGE) Guidelines (Sutherland *et al*, 1996), the number of circulating $CD34^+$ cells was quantified as described (Kikuchi-Taura *et al*, 2006). In brief, blood samples were incubated with phycoerythrin-labeled anti-CD34 antibody, fluorescein isothiocyanate-labeled anti-CD45 antibody, 7-aminoactinomycin-D, and internal control (all of these reagents are from the Stem-Kit, Beckman Coulter, Marseille, France). 7-Aminoactinomycin-D-positive dead cells and CD45-negative cells were excluded, and the number of cells forming a cluster with characteristic $CD34^+$ cells (i.e., low side scatter and low-to-intermediate CD45 staining) was counted. The absolute number of $CD34^+$ cells was calculated using the internal control. In this study, we used a single measurement at the time of entry into the study, on the basis of our previous observation that the level of circulating $CD34^+$ cells is relatively stable (Taguchi *et al*, 2004*a*). For statistical analysis, JMP version 5.1J (SAS Institute Inc, Co, NC, USA) was used. Individual comparisons were performed using a two-tailed, unpaired Students' *t*-test. Statistical comparisons among groups were determined using analysis of variance. Mean $\pm$ s.e. is shown.

Results

Baseline characteristics of the groups are shown in Table 1. In univariate analysis of control subjects, each cerebrovascular risk factor and other treatment showed no significant difference with the number of circulating $CD34^+$ cells (data not shown).

To investigate a possible relationship between circulating $CD34^+$ cells and cognition, the level of circulating $CD34^+$ cells was compared among these groups. Representative fluorescence-activated cell sorting images are shown in Figure 1A (vascular-type) and 1B (Alzheimer's-type). Analysis of variance revealed a significant decrease of $CD34^+$ cells in patients with vascular-type cognitive impairment compared with Alzheimer's-type cognitive impairment ($P<0.001$) and normal subjects ($P<0.001$, Figure 1C).

To investigate further a possible association of circulating $CD34^+$ cells with cognitive impairment, patients with vascular-type impaired cognition were divided into two groups according to their CDR (mild: CDR $=0.5$, $n=22$, mean age $=75.2\pm1.6$ years; moderate-severe: CDR ≥ 1, $n=18$, mean age $=75.3\pm1.5$ years) or MMSE (mild: MMSE ≥ 20, $n=25$, mean age $=74.2\pm1.4$ years; moderate-severe: MMSE <20, $n=15$, mean age $=77.1\pm1.5$ years). The results showed a significant decrease in the level of circulating $CD34^+$ cells in moderate-severe group, based on stratification by either CDR (Figure 1D, $P=0.01$) or MMSE (Figure 1E, $P=0.03$) in patients with vascular-type cognitive impairment. Similar analysis was applied to patients with Alzheimer's-type impaired cognition. They were divided into two groups according to CDR (mild: $n=8$, mean age $=73.0\pm4.7$ years; moderate-severe: $n=15$, mean age $=77.5\pm1.9$ years) or MMSE (mild: $n=12$, mean age $=74.1\pm3.0$ years; moderate-severe: $n=11$, mean age $=77.8\pm2.9$ years). However, in contrast to patients with vascular-type impaired cognition, there was no significant difference observed in patients with Alzheimer's-type cognitive impaired, based on CDR (Figure 1F, $P=0.86$) or MMSE (Figure 1G, $P=0.60$).

Discussion

Our results are consistent with a contribution of circulating $CD34^+$ cells in support of cognitive function, presumably through their positive homeostatic influence on the cerebral circulation in

Table 1 Baseline characteristics

		Cognitive impairment			
	Total	*Vascular-type*	*Alzheimer's-type*	*Control*	*P-value for trend*
n	95	40	23	32	
Age, years	74.9±0.6	75.3±1.1	75.9±2.1	74.2±0.7	0.53
Male gender, *n* (%)	57 (60)	27 (68)	12 (52)	18 (56)	0.46
Risk factor, n (%)					
Hypertension	41 (43)	21 (53)	9 (39)	11 (34)	0.28
Hyperlipidemia	29 (31)	14 (35)	5 (22)	10 (31)	0.53
Diabetes mellitus	9 (9)	5 (13)	1 (4)	3 (9)	0.57
Smoking	20 (21)	10 (25)	6(26)	4 (13)	0.34
Treatment, n (%)					
Ca-channel blocker	30 (32)	15 (38)	6 (26)	9 (28)	0.56
β-Blocker	2 (2)	1 (3)	0 (0)	1 (3)	0.71
ACE inhibitor	4 (4)	3 (8)	1 (4)	0 (0)	0.29
ARB	8 (8)	3 (8)	3 (13)	2 (6)	0.65
Diuretics	6 (6)	2 (5)	1 (4)	3(9)	0.68
Statin	29 (31)	14 (35)	5 (22)	10 (31)	0.54
Aspirin	28 (29)	23 (58)	1 (4)	4 (13)	<0.01
Ticlopidine	11(12)	9 (23)	0 (0)	2 (6)	0.01

ACE, angiotensin-converting enzyme; ARB, angiotensin II receptor blocker.

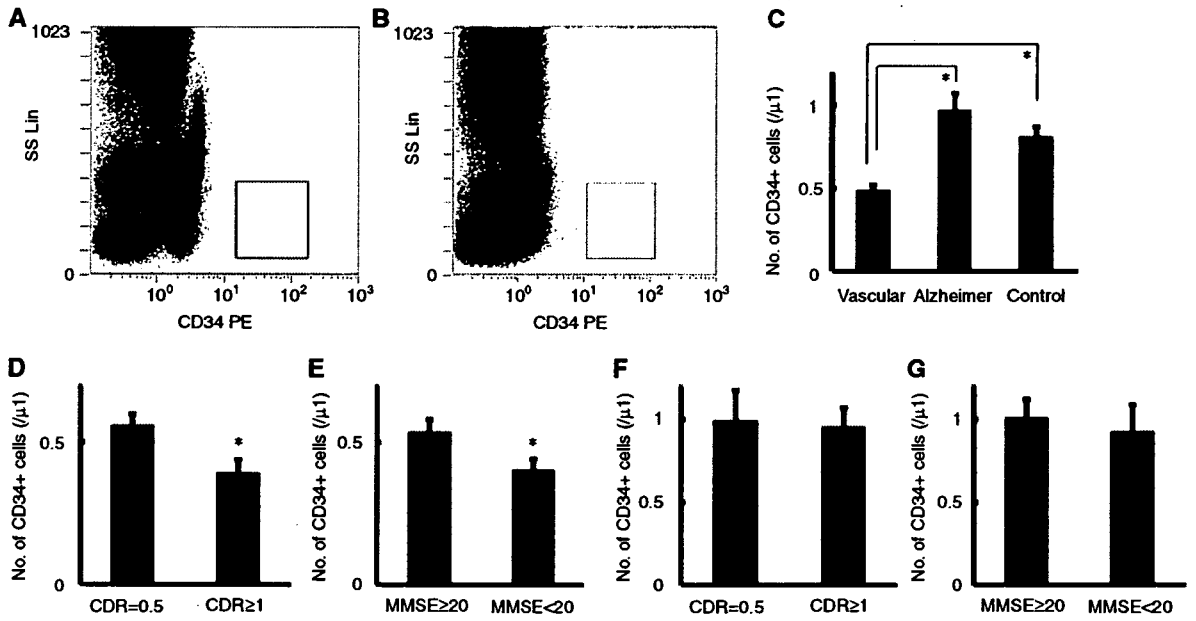

Figure 1 Levels of circulating CD34+ cells and cognitive impairment. (**A** and **B**) After exclusion of 7-AAD-positive dead cells and CD45-negative cells (non-leukocyte), CD34+ cells cluster at low side scatter were clearly observed (**A**, vascular-type; **B**, Alzheimer's-type). (**C**) Analysis of variance revealed a significant decrease in circulating CD34+ cells in patients with vascular-type cognitive impairment compared with normal subjects and individuals with Alzheimer's-type cognitive impairment. In contrast, no significant change in circulating CD34+ cells was observed in patients with Alzheimer's-type cognitive impairment compared with control subjects. (**D** and **E**) In the group of patients with vascular-type cognitive impairment, the level of circulating CD34+ cells was significantly reduced in patients with more severe cognitive impairment compared with the more mildly affected group (**D**, CDR; **E**, MMSE). (**F** and **G**) In contrast, no significant difference was observed in patients with Alzheimer's-type cognitive impairment based on assessment of cognition (**F**, CDR; **G**, MMSE). SS Lin, side-scatter linear scale. $*P < 0.05$.

settings of ischemic stress. Further, these observations suggest a basic difference between the pathobiology of dementia in Alzheimer's disease (without associated cerebral ischemia) and declining cognitive function in patients with ischemic cerebrovascular disorders.

Late onset, sporadic Alzheimer's disease is a heterogeneous disorder (Casserly and Topol, 2004) and the contribution of a vascular factor is still controversial. In contrast to vascular-type cognitive impairment, no significant change (at most, a mild increase) in the level of circulating $CD34^+$ cells was observed in patients with Alzheimer's-type cognitive impairment who had no cerebral ischemia. Consistent with a $CD34^+$ cell-independent mechanism of cognitive decline in Alzheimer's-type impaired cognition, there was no correlation between circulating $CD34^+$ cells and the level of CDR or MMSE. These results suggest that the level of $CD34^+$ cells in the peripheral circulation might provide a useful means of separating dementia with a vascular etiology from dementia associated with nonvascular causes. This is not inconsistent with a previous report indicating decreased levels of $CD34^+$ cells in patients with early Alzheimer's disease that did not exclude patients with coexisting cerebral ischemia (Maler *et al*, 2006). Our findings could have implications for treatment, especially as more modalities become available for patients with declining cognitive function.

The level of circulating endothelial progenitor cells, identified based on positivity for CD34 and kinase insert domain receptor ($CD34^+/KDR^+$ cells), has been correlated with cardiovascular risk factors (Vasa *et al*, 2001) and cardiovascular outcomes (Schmidt-Lucke *et al*, 2005; Werner *et al*, 2005). However, large variations in the levels of $CD34^+/KDR^+$ cells in the latter reports (by ~100-fold between reports; Fadini *et al*, 2006; Werner *et al*, 2005) indicate the need to standardize this measurement. In contrast, in our study, although there was no strong correlation between levels of $CD34^+$ cells and established cardiovascular risk factors and other treatments, probably because of the heterogeneity of our control subjects, the results indicate a close relationship between the overall $CD34^+$ pool and the cognitive impairment with cerebral ischemia. Previous reports have indicated a positive correlation between mobilization of $CD34^+$ cells and improved functional outcome in stroke patients (Dunac *et al*, 2007). Accelerated functional recovery after experimental stroke, because of administration of $CD34^+$ cells (Shyu *et al*, 2006; Taguchi *et al*, 2004*b*), suggests the possible contribution of $CD34^+$ cells in maintenance of brain function during cerebral circulation. Our method for quantification of $CD34^+$ cells is simple, reproducible (Kikuchi-Taura *et al*, 2006), and suitable for screening a broad group of patients at risk for cerebrovascular disorders.

In conclusion, our results indicate that the level of circulating $CD34^+$ cells provides a marker of vascular risk associated with cognitive impairment. Furthermore, differences in the pathobiology of Alzheimer's- and vascular-type cognitive impairment may be mirrored in levels of circulating $CD34^+$ cells in these patient populations.

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Conflict of interest

The authors state no conflict of interest.

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Neuroprotective Effect of Bone Marrow–Derived Mononuclear Cells Promoting Functional Recovery from Spinal Cord Injury

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ABSTRACT

Neural cell transplantation, a new therapeutic strategy for replacing injured neural components and obtaining functional recovery, has shown beneficial effects in animal models. Use of this strategy in human patients, however, requires that a number of serious issues be addressed, including ethics, immunorejection, and the therapeutic time window within which the procedure will be effective. Bone marrow–derived mononuclear cells (BM-MNC) are attractive for transplantation because they can be used as an autograft, can be easily collected within a short time period, and do not have to be cultured. In a rat model of spinal cord injury (SCI), we transplanted BM-MNC at 1 h after SCI at Th 8–9 by injecting them into the cerebrospinal fluid (CSF), and investigated the effect of this on neurologic function. In the acute stage of injury, we found a neuroprotective antiapoptotic effect, with an elevated concentration of hepatocyte growth factor in CSF. At 1 week after transplantation, the Basso–Beattie–Bresnahan locomotor score had increased significantly over its baseline value. In the chronic stage of injury, we observed suppressed cavity formation and functional improvement. We conclude that transplantation of BM-MNC after SCI has a remarkable neuroprotective effect in the acute stage of injury, suppressing cavity formation, and contributing to functional recovery. Our results suggest that transplantation of BM-MNC via the CSF is a potentially effective means of enhancing functional recovery after SCI in humans.

Key words: bone marrow–derived mononuclear cell; cell transplantation; cerebrospinal fluid; neuroprotection; spinal cord injury

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INTRODUCTION

INJURY TO THE CENTRAL NERVOUS SYSTEM (CNS) causes permanent defects in neurologic function. A number of animal experiments involving neural cell transplantation after neural damage, including spinal cord injury (SCI), have been conducted to investigate the replacement of injured neural components and functional recovery after injury to the CNS. Beneficial effects have been reported with Schwann cells (Martin et al., 1996), olfactory ensheathing cells (Doucette, 1995), embryonic stem cells (McDonald et al., 1999), neural stem cells (Ogawa et al., 2002), and choroid plexus ependymal cells (Ide et al., 2001) for such transplantation. However, the transplantation of expanded and/or allogenic cells in human patients requires addressing such issues as immunologic reactivity to such transplantation and the therapeutic time window during which it is effective. Furthermore, clinical trials of neural cell transplantation after stroke have revealed that simple cell transplantation has only a mild therapeutic effect (Kondziolka et al., 2000), probably because of the absence of a favorable environment for reconstitution of the neural system (Taguchi et al., 2004).

Recently, a highly neovascularized environment after injury to the CNS, obtained by hematopoietic stem cell transplantation, was shown to accelerate recovery of neurologic function in a model of stroke (Taguchi et al., 2004). Additionally, clinical trials found that transplantation of bone marrow–derived mononuclear cells (BM-MNC) had beneficial effects on functional recovery after myocardial infarction (Assmus et al., 2002; Strauer et al., 2002). BM-MNC are known to include immature cells such as hematopoietic stem cells and endothelial progenitor cells, and have also been shown to supply multiple growth factors (Rehman et al., 2003) that may protect against secondary neural injury (Widenfalk et al., 2003). We therefore investigated the effect of transplanting BM-MNC in a rat model of SCI, and found that it had a neuroprotective effect that promoted functional improvement.

METHODS

All procedures were performed in accordance with the Guidelines for Animal Experiments of Kyoto University. Quantitative measurements and behavioral tests were performed by investigators unaware of the experimental protocol and the identity of the sections/animals under study.

Preparation of Bone Marrow–Derived Mononuclear Cells

Bone marrow cells were obtained from 8-week-old (adult) male Wistar rats weighing 200–220 g. Cells were harvested after administration of 5–fluorouracil, as described previously (Azizi et al., 1998). BM-MNC were separated by density centrifugation with a commercially available density solution (density, 1.077; Lymphoprep™; Nycomed Pharma, Oslo, Norway; www.nycomed.com) (Tomita et al., 2002). These cells were assessed on the basis of cell surface markers (PharMingen, San Diego, CA), using a fluorescence-activated cell sorter, and the following percentages were found to express the respective markers, which are markers of Thy-1, leukocytes, β1-integrin, c-kit, hematopoietic stem cells, endothelial cells, and macrophages, respectively: 43% CD90, 38% CD45, 74% CD29, 11% CD117, 10% CD34, 24% CD31, and 19% CD11b/c.

To trace cells, BM-MNC were labeled with the fluorescent membrane-intercalating dye PKH67 (green fluorescence; MINI-67; Sigma, St. Louis, MO) (Tomita et al., 2002).

Spinal Cord Injury and Transplantation of BM-MNC

Crush injury was produced in the spinal cords of 40 Wistar rats, all 4 weeks old, by impact with a weight-drop device (NYU Spinal Cord Contusion System; New York University, New York, NY) as previously described (Bai et al., 2003). The rats were anesthetized with pentobarbital 50 mg/kg i.p., laminectomy was done at the level of Th8–9, and injury was achieved by dropping a 10-g weight 2.5 mm in diameter from a height of 12.5 mm (Bai et al., 2003). At 1 h after injury, a cell suspension of 5×10^6 viable BM-MNC, dissolved in 70 μL of Hanks' balanced salt solution (HBSS), was injected with an insulin syringe and stereotaxic coordinates into the fourth ventricles of 20 Wistar rats over a period of 5 min, as previously described (Bai et al., 2003). A control population of 20 rats underwent the same crush injury procedure followed by injection of the same volume of HBSS but without the cell suspension. A total of 18 rats with SCI (n = 9 per group) were used for histologic examination and evaluation in the acute stage of injury. Three rats per group were killed, on days 1, 3, and 7, respectively. A total of six rats with SCI (n = 3 per group) were killed on day 3 for the measurement of cytokines in CSF by enzyme-linked immunosorbent assay (ELISA). Sixteen rats with SCI (n = 8 per group) were tested according to the Basso–Beattie–Bresnahan (BBB) locomotor rating scale (Basso et al., 1995), to evaluate

locomotor function of the hindlimbs before injury and again on days 1, 7, 14, 21, 28, and 35. On day 35, these rats were killed for histologic study.

Preparation of Tissues for Histologic Analysis

A fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer was perfused through the hearts of all rats. Sections of spinal cord tissue 10–μm-thick were subsequently cut longitudinally with a cryostat and mounted on glass slides for observation after staining with hematoxylin and eosin (H&E) or immunohistochemical staining. The following primary antibodies and dilutions were used: mouse monoclonal antibody (Mab) against glial fibrillary acidic protein (GFAP; Sigma) at 1:400; mouse Mab against β-tubulin type III (Sigma) at 1:300; and rabbit polyclonal antibody against von Willebrand Factor (vWF; DAKO, Glostrup, Denmark) at 1:10. A fragment-conjugated goat antimouse antibody (Alexa Fluor; Molecular Probes, Eugene, OR) at a dilution of 1:1000, and a fragment-conjugated goat antirabbit antibody (Alexa Fluor; Molecular Probes) at a dilution of 1:500, were used as second antibodies. To stain for cell nuclei, we used the membrane-impermeant fluorescent DNA-binding dye TO-PRO-3 (Invitrogen, Carlsbad, CA) at a dilution of 1:500.

Evaluation of Apoptotic Cells at the Site of SCI in the Acute Stage of Injury

To quantitate apoptotic cells at the site of injury on day 3 after SCI (n = 3 per group), we used a terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP)–biotin nick-end labeling (TUNEL) assay (DeadEnd™ Fluorometric TUNEL System; Promega) according to the protocol recommended by the manufacturer. We examined five parasagittal sections of injured spinal cord, including the median sagittal section, and compared the numbers of TUNEL-positive cells per field (0.1 mm^2) at high magnification (×40) among the different groups of animals.

Quantification of Protected Axons in the Acute Stage of Injury

To investigate the degree of protection of nerve fibers provided by BM-MNC transplantation in the acute stage of SCI, we used immunohistochemical staining of sections obtained at the injury site, with an anti-β-tubulin type III antibody. We did this on six rats on day 3 after SCI (n = 3 per group) and a further six animals on day 7 (n = 3 per group). We examined a total of 21 sagittal sections obtained from each animal, consisting of one median sagittal section and two sets of 10 consecutive sections cut at intervals of 50 μm from the median plane laterally toward the left (one set) and right (the other set). These sections covered two-thirds of the width of the spinal cord, including whole degenerated nerve fibers. We measured the density of nerve fibers that stained positively for β-tubulin type III at the site of injury within 2.5 mm in the cephalocaudal direction, using the image processing and analysis software NIH Image 1.61. The density of nerve fibers was reported as the percentage of the β-tubulin type III-positive area relative to the entire area of SCI.

Blood Vessels at the Site of Injury in the Acute Stage of Injury

Blood vessels were examined at the site of injury on days 3 and 7. Five parasagittal sections, including the median sagittal section, obtained from the injury site in each animal, were stained for vWF and TO-PRO-3. vWF-positive cells were observed mainly around the center of the injury site. Using NIH Image 1.61 software, we measured the density of vWF-positive cells in sagittal sections centered around and within 2.5 mm of the injury site in the cephalocaudal direction.

ELISA of Cytokines in Cerebrospinal Fluid

We sought to determine whether transplantation of BM-MNC after SCI had an effect on neuroprotective or angiogenic factors in CSF. CSF was obtained from six rats (n = 3 per group) on day 3 after SCI and assayed via ELISA for vascular endothelial growth factor (VEGF) (Immuno-Biological Laboratories, Gunma, Japan), hepatocyte growth factor (HGF) (Institute of Immunology, Tokyo, Japan), and tumor necrosis factor-α (TNF-α) (BioSource International, Camarillo, CA), following the manufacturers' recommended protocols in each assay.

Measurement of Injury-Cavity Volume

The volume of each cavity that developed after SCI was quantified as described previously (Ohta et al., 2004) at 35 days after injury (n = 8 per group). Briefly, horizontal cryostat sections were stained with H&E and examined under a light microscope equipped with a charge-coupled-device (CCD) camera (HC300; Fuji Photo Film Co. Ltd., Tokyo, Japan) (Reyes and Verfaillie, 2001). We used a total of 41 sagittal sections obtained from each animal, consisting of one median sagittal section and two sets of 20 consecutive sections cut at intervals of 50 μm from the median plane laterally toward the left (one set) and right (the other set). These sections covered the entire spinal cord. The area of cavitation in each spinal cord section was measured, as shown in Figure 6G below, using NIH Image 1.61 software. The cavity volume was then calculated by multiplying the average area of cavi-

tation by the depth of the section examined (Takami et al., 2002).

Quantification of Angiogenesis Around the Injury Cavity

We counted the blood vessels around the injury cavity in each rat at 35 days after injury. Vessel density was defined as the number of blood vessels per injury area immediately around the cavity. We investigated the number of blood vessels that stained immunopositively for vWF in the marginal zone extending to within 200 μm of the inner margin of the injury cavity (see Fig. 6I,K below).

Behavioral Analysis

The animals' locomotor activity was evaluated by means of open-field BBB scoring, as described previously (Basso et al., 1995; Wu et al., 2003). Briefly, the behavior of each animal in an open 75 × 120-cm field was observed by two researchers. Scores ranging from 0 (complete paralysis) to 21 (normal gait) were recorded every week after surgery.

Statistical Analysis

Differences in cavity formation and the density of vWF-positive cells on day 35 were analyzed by unpaired Student's *t*-tests. Differences in the concentration of cytokines in CSF, the density of nerve fibers and vWF-positive cells in the acute stage of injury, and BBB score were analyzed with the Mann-Whitney *U*-test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Functional Recovery after Spinal Cord Injury

BBB locomotor scores are shown in Figure 1. The maximum BBB score was 21 before SCI, while the initial BBB score for the spinal cord–injured rats was less than 8. No significant functional improvement was observed at 24 h after SCI in rats that underwent cell transplantation as compared to control rats. However, continuous functional recovery was observed at days 7–35 in rats that underwent BM-MNC transplantation. In contrast, little, if any, functional recovery was observed in control rats. The functional recovery seen in the rats that underwent BM-MNC transplantation was significant.

Transplanted BM-MNC in the Fourth Ventricle and around the Injured Spinal Cord

To trace transplanted BM-MNC labeled with PKH67 (green fluorescence), we examined the fourth ventricle

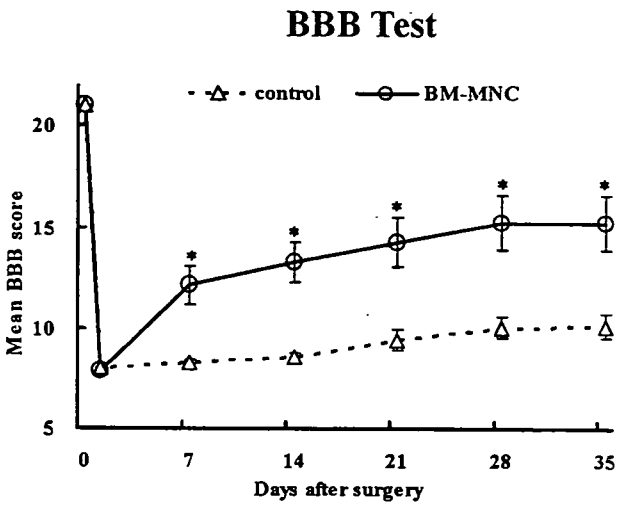

FIG. 1. Administration of BM-MNC promotes rapid improvement of motor function after SCI. To evaluate motor function of the lower limbs, BBB locomotor scores were determined on days 1, 7, 14, 21, 28, and 35 after SCI. Scores range from 0 (complete paralysis) to 21 (normal gait). On day 1 there was no significant difference in the mean score between BM-MNC-transplanted and control groups of animals. However, from day 7 to day 35, the mean scores of rats that underwent BM-MNC transplantation were significantly higher than those of control rats ($n = 8$ per group). $^*p < 0.05$ versus control.

of the rat brain and the injured spinal cord. At 1 day after transplantation, PKH67-labeled cells were observed very close to the choroid plexus in the ventricle (Fig. 2A,B), as well as on the surface of the pia mater of the injured spinal cord (Fig. 2C–E). On day 3, these cells decreased in number, and a piece of transplanted cell was sometimes seen in the ventricle (Fig. 2F), while some transplanted cells were integrated into the pia mater of the injured cord (Fig. 2G–I). On day 7, few, if any, PKH67–labeled cells were observed in the ventricle (Fig. 2J) or around the injured spinal cord (Fig. 2K–M).

Protection of Axons at the Site of Injury in the Acute Stage

To investigate whether BM-MNC have a protective effect on nerve fibers within 1 week after SCI, we performed immunohistochemistry for β-tubulin type III on sections of injured spinal cord on day 3 (Fig. 3A,B) and day 7 (Fig. 3C,D) after injury. In control rats (Fig. 3A,C), the area not stained had often spread to the dorsal side of the spinal cord, where direct contusion injury had occurred. In contrast, in BM-MNC-transplanted rats (Fig. 3B,D), the unstained area was restricted and was smaller than in control rats.

To investigate whether transplantation of BM-MNC protects injured spinal cord from apoptosis, we performed