Fig. 2 – Correlation between ΔCD34+ cells and ΔhsCRP ($r = -0.412$, $p = 0.017$) (a), correlation between ΔCD34+ cells and Δadiponectin ($r = 0.359$, $p = 0.043$) (b), and correlation between ΔCD34+ cells and ΔHbA1c ($r = -0.299$, $p = 0.108$) (c).

1.15 ± 0.57 and 1.15 ± 0.65 cells/μl at 0, 12 and 24 weeks, respectively, $n = 18$). There was no significant difference in the change in CD34+ cell level (ΔCD34+ cells) between 15 mg and 30 mg of pioglitazone (15 mg; 0.07 ± 1.01 vs. 30 mg; 0.14 ± 0.32).

3.4. Factors involved in the stimulation of CD34+ cells

We next investigated which factors were correlated with the stimulation of CD34+ cells. ΔCD34+ cells were significantly correlated with Δhs-CRP in univariate analysis ($r = -0.412$, $p = 0.017$) (Fig. 2a). Further, Δadiponectin correlated with Δ CD34+ cells ($r = 0.359$, $p = 0.043$) (Fig. 2b). On the other hand, change in HbA1c levels (ΔHbA1c) ($r = -0.299$, $p = 0.108$) (Fig. 2c), change in HDL-C levels (ΔHDL-C) ($r = 0.253$, $p = 0.168$) and change in triglyceride levels (Δtriglycerides) ($r = 0.0072$, $p = 0.969$), were not significantly correlated to Δ CD34+ cells.

4. Discussion

Accumulating evidence shows that PPARγ agonists have anti-atherosclerotic actions other than their blood glucose level

reduction effects [7,9]. One recent report showed that pioglitazone treatment could stimulate circulating EPCs in patients with coronary artery disease and normal glucose tolerance [12]. In this study, we demonstrated that pioglitazone treatment also increased circulating CD34+ cells and this effect continued for 24 weeks in type 2 diabetic patients. We studied the effects of pioglitazone on the stimulation of CD34+ cells but not CD34+/KDR+ cells regarded as EPCs. However, these circulating CD34+ cells have the capacity to participate in neovascularization of ischemic tissue. Indeed, their administration enhances the repair of ischemic tissue in ischemic stroke model [13] and improves myocardial circulation in myocardial infarction model [14]. Clinically, circulating CD34+ cell levels were reported to be correlated with cerebral blood flow in hypoperfusion area [6] and formation of collateral vessels in stroke patients [15]. These reports suggest that CD34+ cells may play a role in the maintenance of micro-circulation. One recent clinical trial, PROactive Study, demonstrated that pioglitazone treatment could prevent cardiovascular events including stroke in type 2 diabetic patients [16]. Taken together, it is suggested that the stimulation of CD34+ cells may partly contribute to the preventive effects of pioglitazone on cardiovascular diseases. Our study also demonstrated that pioglitazone treatment increased circulating CD34+ cells in type 2 diabetic patients irrespective of with or without CVD, suggesting that pioglitazone treatment may be useful for primary prevention as well as secondary prevention of diabetic macroangiopathy.

It has been reported that the number of circulating EPCs is inversely correlated with HbA1c levels [3]. Since pioglitazone treatment significantly decreased HbA1c levels and this study did not have control group, we could not exclude the possibility that the stimulation of CD34+ cells was associated with the improvement of glycemic control. However, the results of this study suggest that pioglitazone may be capable of stimulating circulating CD34+ cells independently of glycemic control because ΔCD34+ cells was not positively correlated with ΔHbA1c at levels that achieved statistical significance.

Adipocyte derived factors and inflammation participate in atherogenesis of type 2 diabetic patients. Accumulating evidence show that adiponectin, one of adipocyte derived factors, has anti-atherogenic properties, and hypoadionecti-nemia was reported to be associated with endothelial dysfunction [17]. Pioglitazone treatment decreased hs-CRP levels and increased serum adiponectin levels in metabolic syndrome subjects [8], suggesting that these effects contribute to the anti-atherosclerotic action of pioglitazone. In this study, we also demonstrated that pioglitazone treatment decreased hs-CRP levels and increased serum adiponectin levels in type 2 diabetes patients. Interestingly, ΔCD34+ cells were significantly correlated with Δhs-CRP and Δadiponectin. An in vitro study showed that CRP impaired EPC migration and function [18]. In clinical study, it has been reported that circulating EPCs were inversely correlated to serum interleukin 6 levels [19]. These reports suggested that chronic inflammation may be involved in the regulation of EPCs. One recent clinical study showed that circulating EPCs were positively correlated to serum adiponectin levels in patients with coronary artery disease [20]. Another report showed that

adiponectin treatment increased EPC number and migration [12]. Taken together, it is suggested that the inhibitory effects on chronic inflammation and the effect on adiponectin regulation of pioglitazone may be directly or indirectly involved in the increase of CD34⁺ cells. However, further study is necessary to delineate this hypothesis.

In conclusion, our study demonstrated that pioglitazone treatment increased circulating CD34⁺ cells, suggesting that this effect may at least partly contribute to the anti-atherosclerotic action of pioglitazone.

Conflict of interest

There are no conflicts of interest.

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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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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, 2004b) 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, 2004a), 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.
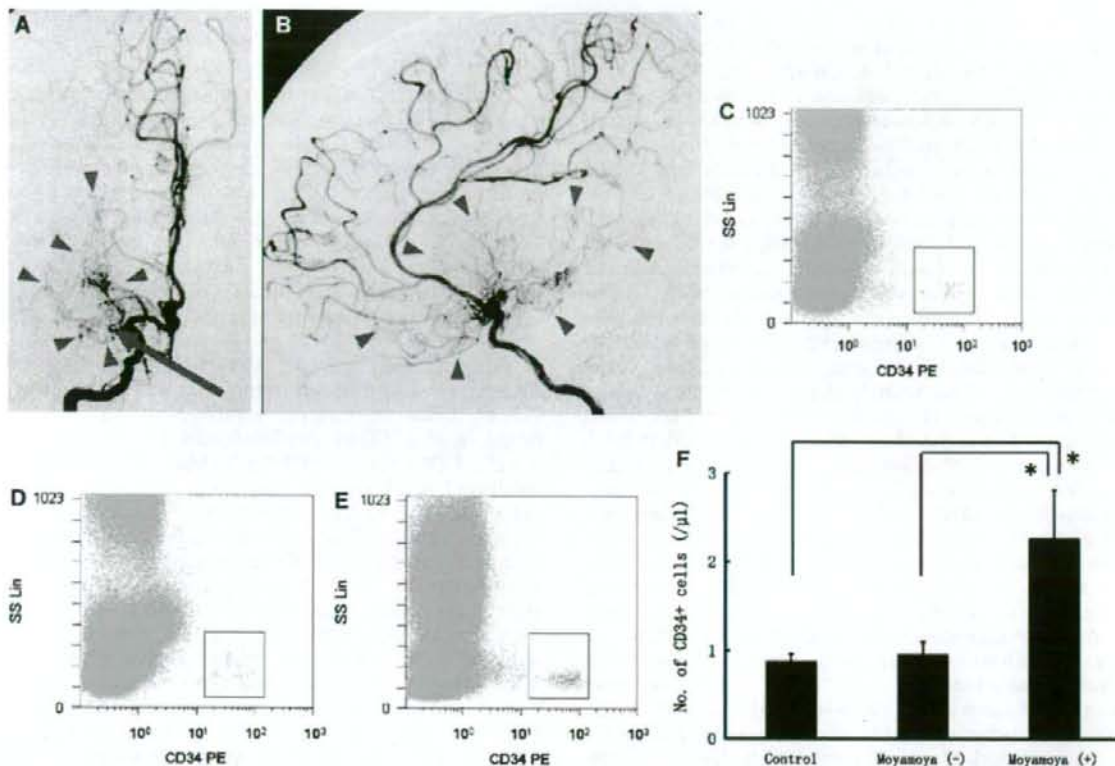
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Figure 1 Increased levels of circulating CD34+ cells in patients with angiographic evidence of moyamoya-like vessels. (A, B) Representative angiogram from a patient with moyamoya-like vessels. Unusually accelerated neovascularization (based on angiographic 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 ± 0.07/μL; moyamoya (−), CD34+ cells = 0.98 ± 0.13/μL; moyamoya (+), CD34+ cells = 2.28 ± 0.53/μL) 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, 2004a). Furthermore, transplantation of CD34+ cells (Taguchi et al, 2004b) and bone marrow cells (Borlongan et al, 2004a,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

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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.

Acknowledgements

Conflict of interest

The authors state no conflict of interest.

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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.

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, 2004a). 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, 2004b).

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 Kosei-nenkin 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, 2004a). 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 ± 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 ± 1.6 years; moderate-severe: CDR ≥ 1, $n = 18$, mean age = 75.3 ± 1.5 years) or MMSE (mild: MMSE ≥ 20, $n = 25$, mean age = 74.2 ± 1.4 years; moderate-severe: MMSE < 20, $n = 15$, mean age = 77.1 ± 1.5 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 ± 4.7 years; moderate-severe: $n = 15$, mean age = 77.5 ± 1.9 years) or MMSE (mild: $n = 12$, mean age = 74.1 ± 3.0 years; moderate-severe: $n = 11$, mean age = 77.8 ± 2.9 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

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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

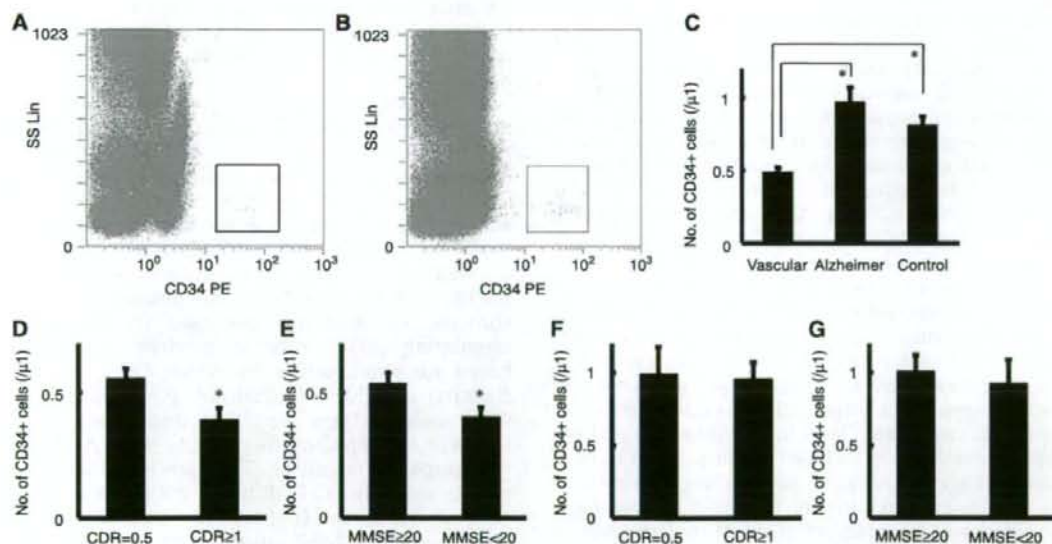ACE, angiotensin-converting enzyme; ARB, angiotensin II receptor blocker.

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, 2004b), 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.

Acknowledgements

Conflict of interest

The authors state no conflict of interest.

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Low circulating CD34 $^{+}$ cell count is associated with poor prognosis in chronic hemodialysis patients

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Circulating CD34-positive (CD34 $^{+}$) cells, a population that includes endothelial progenitor cells, are believed to contribute to vascular homeostasis. Here we determine the prognostic value of CD34 $^{+}$ cell measurements in 216 chronic hemodialysis patients. A total of 43 cardiovascular events and 13 deaths occurred over an average 23 months follow-up in this cohort. A cutoff number for circulating CD34 $^{+}$ cells was determined by receiver operating characteristic curve analysis to maximize the power of the CD34 $^{+}$ cell count in predicting future cardiovascular events. Based on this, 93 patients were categorized as having low and 123 patients as having high numbers of CD34 $^{+}$ cells, determined by flow cytometry at the time of enrollment. Both cumulative cardiovascular event-free survival and all-cause survival were significantly less in the group of patients with low numbers of CD34 $^{+}$ cells. By multivariate analyses, a low level of circulating CD34 $^{+}$ cells was an independent and significant predictor for both cardiovascular events and all-cause mortality. Our study shows that a reduced number of circulating CD34 $^{+}$ cells is significantly associated with vascular risks and all-cause mortality in patients on chronic hemodialysis. These cells may be a useful biomarker.

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It is well known that cardiovascular disease (CVD) is the leading cause of death among chronic hemodialysis (HD) patients.[1] However, the traditional risk factors (including hypertension and increased low-density lipoprotein (LDL) cholesterol) and uremia-related risk factors (hemodynamic overload, abnormal calcium metabolism, and so on) do not fully explain the extent and severity of CVD observed among this population.[2-4]

Growing evidence suggests that bone-marrow-derived circulating progenitor cells, including CD34-positive (CD34 $^{+}$) cells, contribute to vascular homeostasis in adults,[5,6] not only as a pool of endothelial progenitor cells (EPCs) but also as the source of growth/angiogenesis factors.[7] The level of EPCs has been shown to predict future events and deaths from CVD among patients with coronary artery disease (CAD).[8,9] We have also shown that a lower number of circulating CD34 $^{+}$ cells is significantly associated with vascular risks.[10-12]

Several researchers have demonstrated that patients on dialysis had a lower EPC count than did control subjects.[13-16] However, there is no definite consensus concerning the absolute number of EPCs in HD patients and its relationship with the prognosis.

These observations prompted us to conduct the present study. We hypothesize that circulating CD34 $^{+}$ cells accelerate the repair of the dysfunctional endothelium, and that a reduced number of these cells results in poor outcomes in chronic HD patients. In this study, we measured the levels of circulating CD34 $^{+}$ cells and prospectively analyzed first CV (cardiovascular) events and deaths by any cause.

RESULTS

Relationship between CD34 $^{+}$ cell count and baseline variables

Out of 216 chronic HD patients who participated in this study, none was lost to follow-up, and none received kidney transplants. The number of circulating CD34 $^{+}$ cells ranged from 0.07 to 2.17/µl (median, 0.41/µl), with a mean (± s.d.)

of $0.49 \pm 0.32/\mu l$. The age of the patients was 65 ± 11 years (range, 35–94 years). A multivariate regression analysis revealed that factors positively associated with the CD34⁺ cell count were gender (male), elevated white blood cell count, and high serum albumin, whereas the negatively associated factors were advanced age and smoking (Table 1).

Baseline clinical variables for the low/high CD34⁺ groups

To further clarify the importance of CD34⁺ cells, we then determined a cutoff value. A receiver operating characteristic (ROC) curve analysis showed $0.37/\mu l$ to be the value (area under the curve = 0.707) to maximize the power of circulating CD34⁺ cell levels as a predictor of a CV event (Figure 1). The patients were categorized into two groups

Table 1 | Relationship between CD34⁺ cell count and baseline variables on multivariate regression analysis

	β	P-value
Male	0.197	**0.021**
Age	-0.157	**0.039**
Duration of HD	0.001	0.99
Diabetes	0.054	0.50
Hypertension	-0.079	0.24
Smoking	-0.294	**0.0001**
Body mass index	0.043	0.57
History of CVD	-0.035	0.61
Hemoglobin	-0.124	0.54
WBC	0.300	**<0.0001**
Albumin	0.148	**0.049**
HDL cholesterol	-0.036	0.61
LDL cholesterol	-0.058	0.39
Ca × Pi	0.092	0.19
Intact PTH	0.197	0.34
C-reactive protein	0.002	0.97
KT/V$_{urea}$	0.080	0.36

Ca × Pi, calcium-phosphate product; CVD, cardiovascular disease; HD, hemodialysis; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PTH, parathyroid hormone; KT/V$_{urea}$, urea clearance × time normalized by total body water; WBC, white blood cell. P-values <0.05 are shown in bold.
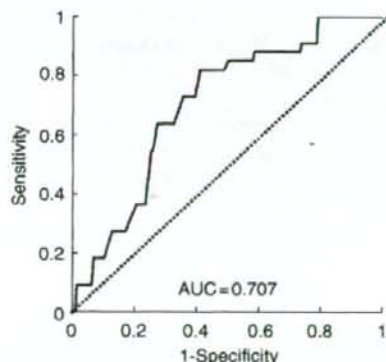

Figure 1 | ROC Curve Analysis. A ROC curve analysis was performed to determine a cutoff value for circulating CD34⁺ cell count. The result showed $0.37/\mu l$ to be the value (area under the curve = 0.707) to maximize the power of the CD34⁺ cell count in predicting a future CV event.

according to the cell count at the time of enrollment: the low CD34⁺ group representing 93 patients with circulating CD34⁺ cell counts less than $0.37/\mu l$ (a mean of $0.23 \pm 0.08/\mu l$) and the high CD34⁺ group representing 123 patients with counts of $0.37/\mu l$ or greater (a mean of $0.69 \pm 0.30/\mu l$). The baseline characteristics are shown in Table 2. Patients in the low CD34⁺ group were older (68 ± 9 years) than those in the high CD34⁺ group (62 ± 11 years) ($P < 0.0001$). White blood cell counts were lower in the former group than in the latter. Body mass index and calcium-phosphate product (Ca × Pi) levels were also lower in the patients of the low CD34⁺ group. Gender, duration of HD, smoking, incidence of diabetes, history of CVD, and the use of erythropoietin, were comparable between the two groups. Medications commonly used to decrease CVD, including statins, angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, calcium antagonists, β-blockers were also comparable (Table 2).

Incidence of CV events and all-cause deaths

Table 3 shows the incidence of outcomes. In the low CD34⁺ group, a CV event occurred in 27 out of 93 patients (29%) and 5 patients died from CVD (5.4%). In the high CD34⁺ group, a CV event occurred in 16 (13%) and only 1 patient died of CVD. Concerning death by any cause, 10 patients (10.8%) died in the low CD34⁺ group, whereas three (2.4%) died in the high CD34⁺ group (Table 3). The cumulative CV event-free survival was significantly lower in the low CD34⁺ group (70.6%) than the high CD34⁺ group (86.8%) ($P = 0.0034$; Figure 2). The cumulative all-cause survival was also lower in the low CD34⁺ group (89.2%) than in the high CD34⁺ group (97.5%) ($P = 0.012$; Figure 3).

Factors associated with CV events

Factors associated with CV events are shown in Table 4. In univariate analyses, the incidence of CV events was significantly associated with a level of circulating CD34⁺ cells lower than $0.37/\mu l$ (hazard ratio (HR), 2.90; 95% CI, 1.45–5.81; $P = 0.0026$), advanced age (HR, 1.03; 95% CI, 1.01–1.06; $P = 0.021$), a history of CVD (HR, 7.85; 95% CI, 2.43–12.50; $P = 0.0045$), a low level of serum albumin (HR, 0.24; 95% CI, 0.08–0.67; $P = 0.0066$), or a high level of LDL cholesterol (HR, 1.02; 95% CI, 1.01–1.03; $P = 0.0048$). In a multivariate regression analysis, a level of circulating CD34⁺ cells lower than $0.37/\mu l$ (HR, 2.23; 95% CI, 1.09–4.58; $P = 0.028$), a history of CVD (HR, 6.19; 95% CI, 1.63–9.90; $P = 0.014$), a low level of serum albumin (HR, 0.33; 95% CI, 0.11–0.99; $P = 0.049$), and a high level of LDL cholesterol (HR, 1.02; 95% CI, 1.01–1.03; $P = 0.011$) were identified as independent predictors of CV events among chronic HD patients (Table 4).

Factors associated with all-cause death

Factors associated with all-cause deaths are shown in Table 5. In univariate analyses, all-cause death was significantly associated with a level of circulating CD34⁺ cells lower than

Table 2 | Baseline characteristics of the low/high CD34+ groups

	All patients (n=216)	Low CD34+ group (CD34+ < 0.37/μl) (n=93)	High CD34+ group (CD34+ > 0.37/μl) (n=123)	P-value
Male (%)	122 (56.4)	50 (53.7)	72 (58.5)	0.48
Age (years)	65 ± 11	68 ± 9	62 ± 11	< 0.0001
Duration of HD (years)	8.1 ± 7.1	8.7 ± 7.7	7.8 ± 6.7	0.39
Diabetes (%)	105 (48.6)	44 (47.3)	61 (49.5)	0.73
Hypertension (%)	157 (72.7)	67 (72.0)	90 (74.3)	0.7
Smoking (%)	64 (29.6)	32 (34.7)	32 (26.0)	0.16
Body mass index	20.7 ± 3.2	19.9 ± 2.7	21.4 ± 3.4	0.0008
History of CVD (%)	94 (43.5)	46 (49.5)	48 (39.0)	0.12
CD34+ cells (/μl)	0.49 ± 0.32	0.69 ± 0.30	0.23 ± 0.08	0.0001
Hemoglobin (g/100 ml)	10.6 ± 1.1	10.3 ± 1.1	10.5 ± 1.3	0.11
WBC (10³/μl)	5.9 ± 1.9	5.4 ± 1.6	6.4 ± 1.9	< 0.0001
Albumin (mg/100 ml)	3.6 ± 0.3	3.5 ± 0.3	3.6 ± 0.3	0.11
HDL cholesterol (mg/100 ml)	41 ± 13	42 ± 12	40 ± 14	0.3
LDL cholesterol (mg/100 ml)	77 ± 27	75 ± 27	76 ± 26	0.93
Ca × Pi	49.7 ± 11.8	47.2 ± 11.6	51.7 ± 11.7	0.0062
Intact PTH (ng/ml)	122 ± 114	116 ± 130	126 ± 101	0.52
C-reactive protein (mg/100 ml)	0.42 ± 0.86	0.45 ± 0.78	0.36 ± 0.83	0.41
KT/V$_{urea}$	1.46 ± 0.23	1.49 ± 0.24	1.44 ± 0.22	0.1
Erythropoietin (U/kg)	93 ± 66	99 ± 69	86 ± 64	0.5
Statins (%)	27 (12.5)	10 (10.8)	17 (13.8)	0.49
ARB (%)	87 (40.3)	36 (38.7)	51 (41.5)	0.68
ACEI (%)	38 (17.6)	15 (16.1)	23 (18.7)	0.62
Ca antagonist (%)	133 (61.6)	60 (64.5)	73 (59.4)	0.44
β-Blocker (%)	45 (20.8)	24 (25.8)	21 (17.1)	0.12

ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; Ca × Pi, calcium-phosphate product; CVD, cardiovascular disease; HD, hemodialysis; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PTH, parathyroid hormone; KT/V$_{urea}$, urea clearance × time normalized by total body water; WBC, white blood cell. P-values < 0.05 are shown in bold.

Table 3 | First cardiovascular events and all-cause death during follow-up period

	All patients (n=216)	Low CD34+ group (CD34+ < 0.37/μl) (n=93)	High CD34+ group (CD34+ > 0.37/μl) (n=123)
Total number of CV events (%)	43 (19.9)	27 (29.0)[a]	16 (13.0)
Nonfatal			
Coronary artery disease	27	16	11
PCI	25	15	10
CABG	2	1	1
Stroke	5	3	2
PAD	5	3	2
Fatal			
Congestive heart failure	3	3	0
Stroke	1	0	1
Myocardial infarction	1	1	0
Valve disease	1	1	0
Total number of death (%)	13 (6.0)	10 (10.8)[b]	3 (2.4)
Congestive heart failure	5	3	2
Stroke	3	2	1
Myocardial infarction	1	1	0
Valve disease	1	1	0
Infection	2	2	0
Ischemic colitis	1	1	0

CV, cardiovascular; CABG, coronary artery bypass graft; PAD, peripheral artery disease; PCI, percutaneous coronary intervention.
[a]P=0.0032 vs high CD34+ group.
[b]P=0.012 vs high CD34+ group.

0.37/μl, advanced age, a low body mass index, or a low level of serum albumin. In a multivariate regression analysis, a level of circulating CD34+ cells lower than 0.37/μl (HR, 5.02; 95% CI, 1.08–23.25; $P = 0.040$), advanced age (HR, 1.09; 95%

CI, 1.02–1.15; $P = 0.0082$), and a low level of serum albumin (HR, 0.16; 95% CI, 0.01–0.44; $P = 0.0018$) were identified as independent predictors of all-cause death among chronic HD patients (Table 5).
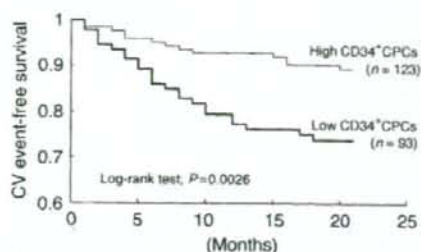
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Figure 2 | Cumulative CV event-free survival in the low/high CD34+ groups. Patients were categorized into the low CD34+ group (CD34+ cells <0.37/µl, n=93) or the high CD34+ group (CD34+ cells >0.37/µl, n=123) using the cutoff value determined by ROC curve analysis. CV event-free survival was defined as the period before the first CV event.
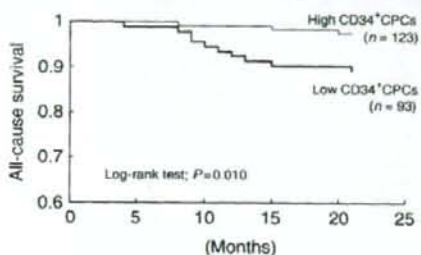

Figure 3 | Cumulative all-cause survival in the low/high CD34+ groups. All-cause survival was analyzed for the low CD34+ group (CD34+ cells <0.37/µl, n=93) and the high CD34+ group (CD34+ cells >0.37/µl, n=123).

Stability of the CD34+ cell count

At 3 years after enrollment, 20 HD patients were randomly selected from among the participants, and the second and the third measurements of CD34+ cell count were conducted at an interval of 1 month. A good correlation was observed between these two additional measurements, which were then averaged for each of the 20 patients. When this value was compared to the first measurement conducted upon enrollment, a correlation was observed, suggesting that the CD34+ cell count is stable over a 3-year period (Figure 4).

DISCUSSION

Recent studies have shown that a low number of EPCs is associated with a poor CV outcome among non-HD patients who had CAD.[8,9] The present study clearly demonstrated that a reduced number of CD34+ cells in the peripheral blood was significantly associated with future CV events as well as all-cause deaths in chronic HD patients. Of importance is the fact that the absolute number of CD34+ cells obtained from chronic HD patients was much lower (0.49 ± 0.32/µl) than that obtained from patients with cerebrovascular disease (1.1 ± 0.31/µl) or control subjects (1.6 ± 0.2/µl).[11]

We measured the levels of CD34+ cells but not the levels of EPCs, which are positive for both CD34 and kinase insert domain receptor. We have shown that circulating CD34+ cell levels are associated with ischemic stroke,[11] brain natriuretic peptide level in type 2 diabetes patients,[10] and vascular risk associated with cognitive impairment.[12] We have also shown that administration of CD34+ cell ameliorates cerebral

Table 4 | HR for cardiovascular events on Cox proportional hazard models

	Univariate		Multivariate	
	HR (95% CI)	P-value	HR (95% CI)	P-value
CD34+ cells <0.37/µl	2.90 (1.45–5.81)	0.0026	2.23 (1.09–4.58)	0.028
Male	0.87 (0.39–1.45)	0.4		
Age (years)	1.03 (1.01–1.06)	0.021	1.01 (0.97–1.04)	0.77
Duration of HD (years)	1.01 (0.95–1.04)	0.93		
Diabetes	1.76 (0.90–3.43)	0.099		
Hypertension	1.05 (0.54–2.17)	0.89		
Smoking	1.38 (0.74–2.59)	0.31		
Body mass index	0.90 (0.80–1.01)	0.075		
History of CVD	7.85 (2.43–12.50)	0.0045	6.19 (1.63–9.90)	0.014
Hemoglobin (g/100 ml)	0.93 (0.72–1.21)	0.57		
WBC (10^3/µl)	1.02 (0.87–1.19)	0.81		
Albumin (mg/100 ml)	0.24 (0.08–0.67)	0.0066	0.33 (0.11–0.99)	0.049
HDL cholesterol (mg/100 ml)	0.99 (0.97–1.02)	0.59		
LDL cholesterol (mg/100 ml)	1.02 (1.01–1.03)	0.0048	1.02 (1.01–1.03)	0.011
Ca × Pi	1.01 (0.92–1.11)	0.74		
Intact PTH (ng/ml)	1.00 (0.99–1.01)	0.37		
C-reactive protein (mg/100 ml)	1.18 (0.89–1.56)	0.26		
KT/V$_{urea}$	0.81 (0.22–2.98)	0.75		
Erythropoietin (U/kg)	1.00 (0.99–1.01)	0.34		
Statins	1.20 (0.46–3.23)	0.7		
ARB	0.96 (0.38–8.40)	0.93		
ACEI	1.47 (0.63–3.45)	0.37		
Ca antagonist	0.91 (0.57–2.11)	0.78		
β-Blocker	1.08 (0.42–2.02)	0.84		

ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; Ca × Pi, calcium-phosphate product; CVD, cardiovascular disease; HD, hemodialysis; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PTH, parathyroid hormone; KT/V$_{urea}$, urea clearance × time normalized by total body water; WBC, white blood cell. Multivariate model includes variable with P<0.05 by univariate analysis. P-values <0.05 are shown in bold.

Table 5 | HR for all-cause mortality on Cox proportional hazard models

	Univariate		Multivariate	
	HR (95% CI)	P-value	HR (95% CI)	P-value
CD34⁺ cells < 0.37/µl	6.17 (1.34–18.57)	**0.019**	5.02 (1.08–23.25)	**0.04**
Male	0.92 (0.28–3.02)	0.88		
Age (years)	1.11 (1.04–1.18)	**0.0009**	1.09 (1.02–1.15)	**0.0082**
Duration of HD (years)	1.03 (0.94–1.10)	0.63		
Diabetes	1.86 (0.54–6.32)	0.32		
Hypertension	1.03 (0.27–3.86)	0.96		
Smoking	1.32 (0.43–4.04)	0.62		
Body mass index	0.73 (0.57–0.92)	**0.0087**	0.79 (0.62–1.01)	0.054
History of CVD	2.35 (0.72–7.69)	0.15		
Hemoglobin (g/100 ml)	0.86 (0.52–1.42)	0.56		
WBC (10³/µl)	1.07 (0.83–1.37)	0.62		
Albumin (mg/100 ml)	0.19 (0.08–0.58)	**0.0006**	0.16 (0.01–0.44)	**0.0018**
HDL cholesterol	0.99 (0.97–1.04)	0.69		
LDL cholesterol	1.01 (0.99–1.03)	0.33		
Ca × Pi	1.02 (0.98–1.07)	0.75		
Intact PTH (ng/ml)	1.00 (0.99–1.02)	0.48		
C-reactive protein	1.04 (0.51–2.08)	0.91		
KT/V_urea	0.56 (0.07–4.78)	0.6		
Erythropoietin	1.01 (0.99–1.02)	0.34		
Statins	1.80 (0.38–8.40)	0.45		
ARB	0.32 (0.07–1.51)	0.66		
ACEI	0.60 (0.08–4.80)	0.63		
Ca antagonist	0.73 (0.46–4.08)	0.56		
β-Blocker	0.57 (0.17–1.87)	0.36		

ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; Ca × Pi, calcium-phosphate product; CVD, cardiovascular disease; HD, hemodialysis; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PTH, parathyroid hormone; KT/V_urea, urea clearance × time normalized by total body water; WBC, white blood cell. Multivariate model includes variable with $P < 0.05$ by univariate analysis. P-values < 0.05 are shown in bold.
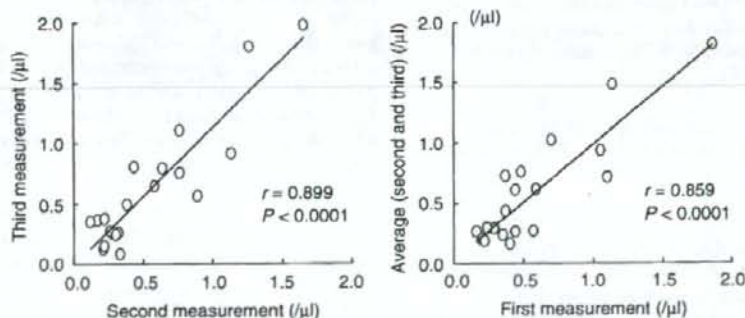

Figure 4 | Stability of CD34⁺ Cell Count. Among 20 of the participants, CD34⁺ cell counts were measured three times. The left graph shows the relationship between the second and the third measurements performed 3 years after enrollment, at an interval of 1 month. The right graph shows the relationship between the first measurement and the average of the second and the third measurements.

ischemia in mice.[17] In humans, injection of CD34⁺ cells derived from peripheral blood improved the ischemia of the lower limbs.[18] These results support the hypothesis that circulating CD34⁺ cells are involved in the pathogenesis of CVD. Indeed, a recent study by Fadini et al.[19] demonstrates that the levels of CD34⁺ cells predict cardiovascular outcome more strongly than the levels of EPCs. In addition, previous studies have shown that our method for quantifying circulating CD34⁺ cells is simple and reproducible.[10–12,20,21] Moreover, the present study demonstrated that the CD34⁺ cell count is relatively stable over 3 years. Therefore, the measurement of CD34⁺ cells would be useful for screening a high-risk population such as chronic HD patients.

In the present study, we set the death from any cause, not the death from CVD, as the primary end point. We presumed that the number of CV deaths would be too small to draw a definite conclusion. Moreover, we thought that all-cause death may be more suitable for this study because it would be difficult to identify all deaths by CVD in chronic HD patients. In fact, both patients whose recorded cause of death was infection had suffered strokes, and one had severe peripheral vascular disease. Although these two patients did not die from CVD directly, their atherosclerotic vascular diseases likely contributed to their outcomes.

We tried to enroll all of the out patients undergoing HD in the clinic. Therefore, it is not likely that there was a selection bias.

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The major limitation of the present study would be the sample size. It is known that cardiovascular events and all-cause mortality in Japanese are significantly lower than those among Caucasians and African Americans.[4,22] In the present study, only 13 (6%) out of 216 patients died during the follow-up period. To draw a more definite conclusion, a larger population would need to be studied. Moreover, a previous study has shown that HD patients who had an elevated number of circulating endothelial cells were at risk for a CV event probably because endothelial cells had become detached from the injured endothelium.[23] A study to clarify the relationship between circulating endothelial cells and CD34 + cells would be worth pursuing.

In summary, the present study demonstrates that a low level of circulating CD34$^+$ cells predicts both future CV events and all-cause deaths in chronic HD patients. Pending further studies, we propose that a single measurement of CD34$^+$ cells taken from the peripheral blood is useful in identifying chronic HD patients at high risk.

MATERIALS AND METHODS
Study population
All the outpatients who underwent maintenance HD therapy in Nagoya Kyoritsu Hospital were eligible for this study. Patients who experienced a vascular event within 30 days of measurements, and those with evidence of infection and/or malignant disease were excluded. A total of 216 chronic HD patients were enrolled in this study between March 2005 and May 2005. The study was performed according to the guidelines of the Declaration of Helsinki Principles, and all patients gave their informed written consent to participate in this study, which was approved by the local ethics committee.

Follow-up
Clinical follow-up was conducted until April 2007. The data for all participants were obtained from medical records kept during the clinical follow-up period. Patients were adequately managed with regular HD treatment three times a week, and routine screening tests for CVD were performed as described previously.[4] In brief, a standard electrocardiogram and chest X-ray were taken every month, and an echocardiogram and a treadmill exercise test were performed at least once a year. When a patient showed abnormal findings in these routine tests or symptoms of CAD during the follow-up period, coronary angiography was adequately and promptly performed.

Previous events, CV events, and causes of deaths
The classification of previous events was made on the basis of medical records and personal interviews. Causes of deaths were determined by examination of hospital records, autopsy reports, and medical files of the patients' general practitioners. CV events were defined as incidents requiring hospitalization due to CVD including CAD, stroke, and peripheral artery disease, or incidents requiring hospitalization for the purpose of percutaneous coronary intervention or coronary artery bypass graft. Deaths from CVD including CAD, congestive heart failure, stroke, arrhythmia, or valve disease were also defined as CV events. End points were the first CV event and all-cause death.

Preparation of blood samples and quantification of circulating CD34$^+$ cells in peripheral blood
Using a modification of the International Society of Hematotherapy and Graft Engineering guidelines,[18] the precise number of circulat-

ing CD34$^+$ cells was quantified as described (the cumulative intraassay coefficient of variation was about 7%).[11,19] Briefly, 0.2 ml of heparinized peripheral blood drawn from the arterial-venous shunt vessel before starting HD was incubated with antibodies to CD34 and CD45, and 7AAD (Stem count kit; Beckman Coulter, Fullerton, CA, USA) followed by lysis of red blood cells. After adding internal controls (Stem count kit; Beckman Coulter), samples were concentrated by centrifugation and analyzed by Coulter CYTOMICS FC500 and XL System II software (Beckman Coulter). Using this method, CD34$^+$ cells were clearly observed as a discrete population of CD34$^+$/CD45^{mild}/7AAD$^-$ cells.

Additional measurements of circulating CD34$^+$ cells. To examine the stability of the CD34$^+$ cell count, additional measurements were performed. In May 2008, 20 patients were randomly selected from among the participants. The first measurement had been performed upon enrollment. A second blood sample was taken in May 2008, and a third sample was taken 1 month later. The association between the second and the third measurements was analyzed. Then, these two values were averaged for each of the 20 patients, and compared to the first measurement.

Statistical analyses
First, the relationship between the CD34$^+$ count and other baseline clinical valuables was studied by a multivariable regression analysis. Next, a cutoff number of circulating CD34$^+$ cells (0.37/μl) was determined by ROC analysis (area under the curve = 0.707) to maximize the power of the CD34$^+$ cell count in predicting future CV events. The patients were then categorized into two groups according to the cell count at the time of enrollment; a low CD34$^+$ group (CD34$^+$ cells < 0.37/μl, $n=93$) or a high CD34$^+$ group (CD34$^+$ cells > 0.37/μl, $n=123$). The cumulative survival rates in each group were estimated by the Kaplan–Meier method, and the differences in survival rates between groups were evaluated by log-rank (Mantle–Cox) method. Student's t-test was used for comparison of quantitative data between the groups. HRs and confidence intervals were calculated for each factor by a Cox univariate analysis, and prognostic factors to predict cardiac events or all-cause death were determined. All the prognostic valuables with $P < 0.25$ were entered into a Cox multivariable analysis to determine independent predictors. All the analyses were performed using a software program, StatView 5.0 (SAS Institute, Cary, NC, USA). Data were expressed as the mean value ± s.d. Differences were considered significant when P-value was < 0.05.

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Pathophysiology/Complications

BRIEF REPORT

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. 1A), 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. 1B). 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 ($r = 0.59$, $P = 0.001$).
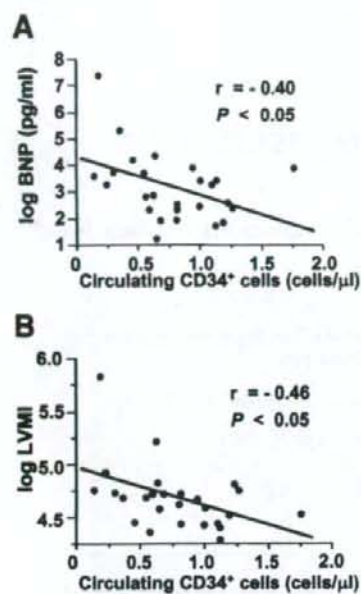
In multiple regression analysis, the

A

B

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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