Decreased levels of circulating CD34\(^+\) cells are associated with coronary heart disease in Japanese patients with type 2 diabetes

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**ABSTRACT**

**Aims/Introduction:** Circulating progenitor cells, including CD34 positive (CD34\(^+\)) cells, play a key role in neovascularisation and the maintenance of vascular endothelial function. Several lines of evidence show an association between decreased levels of circulating CD34\(^+\) cells and cardiovascular disease. However, the contribution of circulating CD34\(^+\) cells to the occurrence of cardiovascular events in diabetic patients remains unclear.

**Materials and Methods:** In the present study with a median follow up of 4.6 years, we analyzed the level of circulating CD34\(^+\) cells in 192 patients with type 2 diabetes. The outcome variables were coronary heart disease (CHD) events (cardiovascular death, unstable angina, myocardial infarction, percutaneous coronary intervention or coronary artery bypass grafting) and cerebrovascular disease events (cerebral infarction, cerebral hemorrhage or transient ischemic attack).

**Results:** Decreased levels of circulating CD34\(^+\) cells were associated with a significantly higher incidence of CHD based on Kaplan–Meier analysis \((P = 0.0052)\). After adjusting for age, sex, dyslipidemia, hypertension, glycated hemoglobin, history of cardiovascular disease, body mass index, and statin and renin angiotensin system inhibitors use, decreased levels of CD34\(^+\) cells were significantly associated with the incidence of CHD events (hazard ratio of low tertile 2.61, 95% confidence interval 1.22–5.96; \(P = 0.013\), reference; high tertile).

**Conclusions:** Decreased levels of circulating CD34\(^+\) cells might predict CHD events in patients with diabetes, and this could be useful for identifying patients with diabetes at high risk of cardiovascular events.

**INTRODUCTION**

The incidence of diabetes mellitus has been rapidly increasing all over the world, and previous epidemiological studies have shown that diabetes is associated with a markedly increased risk of death as a result of cardiovascular disease.

Endothelial dysfunction plays a key role in the progression of atherosclerosis, and circulating bone marrow-derived endothelial progenitor cells (EPCs) participate in the repair of vascular endothelial cells and thus the maintenance of endothelial function. In patients with diabetes, decreases in and dysfunction of circulating EPCs have been reported, suggesting that circulating EPCs contribute to macrovascular complications of diabetes\(^1\).

Circulating immature bone marrow-derived cells contribute to the maintenance of vascular homeostasis and repair, and play an important role in the maintenance of vascular endothelial function. CD34\(^+\) cells, a type of immature circulating bone marrow-derived cell, contribute to the maintenance of the vasculature, as part of a pool of EPCs, and as a source of growth and angiogenesis factors\(^2\). Indeed, we previously reported that the administration of CD34\(^+\) cells enhances the repair of ischemic tissues in a mouse model of stroke\(^3\). We also reported that levels of circulating CD34\(^+\) cells are inversely associated with plasma B-type natriuretic peptide levels\(^4\). Furthermore, a
previous report showed that the CD34+ cell level of subjects with diabetes was lower than subjects with normal glucose tolerance5. However, the contribution of circulating CD34+ cells to cardiovascular events in patients with diabetes remains unclear.

Therefore, we investigated whether the level of circulating CD34+ cells correlates with coronary heart disease (CHD) and cerebrovascular disease (CVD) through a prospective analysis of CVD outcomes during a follow-up period of 2–9 years.

METHODS

Study Participants

We randomly recruited 192 patients with type 2 diabetes (125 men and 67 women, age 64 ± 10 years, duration of diabetes 14 ± 10 years) at a single center between August 2004 and September 2006. Each participant gave written informed consent, and the study was approved by the local ethics committee. Type 2 diabetes was diagnosed according to the Japanese Diabetes Society (JDS) criteria; that is, fasting blood glucose ≥126 mg/dL, glycated hemoglobin (HbA1c) ≥6.5% or casual blood glucose ≥200 mg/dL, and usually not treated with insulin during the first year after diagnosis. The value for HbA1c (%) is estimated as a National Glycohemoglobin Standardization Program equivalent value (%), calculated as HbA1c (%) = HbA1c (JDS; %) +0.3% if HbA1c (JDS) <5, +0.4% if 5 ≤ HbA1c (JDS) < 10, or +0.5% if 10 ≤ HbA1c (JDS), according to the relationship between HbA1c (JDS; %) measured by the previous Japanese standard substance and measurement methods and HbA1c (National Glycohemoglobin Standardization Program)6. Hypertension was defined as systolic blood pressure (SBP) ≥140 mmHg or diastolic blood pressure (DBP) ≥90 mmHg, or both, or the use of antihypertensive medications. Dyslipidemia was defined as serum total cholesterol ≥5.69 mmol/L, triglycerides (TG) ≥1.03 mmol/L, high-density lipoprotein cholesterol ≤1.03 mmol/L or use of lipid-lowering agents.

Definition of Cardiovascular Event

The study outcome was time to first or first recurrence of cardiovascular events. A CHD event was defined as hospitalization for unstable angina, myocardial infarction, percutaneous coronary intervention or coronary artery bypass grafting and cardiovascular death. A CVD event was defined as hospitalization for cerebral infarction, cerebral hemorrhage or transient ischemic attack. Incident events were determined from the diagnoses listed on the discharge summaries according to the International Classification of Diseases, version 10.

Quantification of CD34+ Cells

After 12-h of fasting, 3 mL of heparinized peripheral blood were obtained and CD34+ cells were measured. The precise number of circulating CD34+ cells was quantified as we described previously7. We evaluated circulating CD34+ cells with a Stem-Kit™ (BeckmanCoulter, Marseille, France) according to the manufacturers’ protocols. These protocols are based on International Society of Hematotherapy and Graft Engineering (ISHAGE) Guidelines8, and are frequently used for quantification of CD34+ cells mobilized into the peripheral blood. To increase the reproducibility of CD34+ cell counts, the protocol of the Stem-Kit was modified as follows: the blood sample volume, antibodies and lysing solution were doubled. After adding 30 µL of internal control (Stem count: BeckmanCoulter), samples were centrifuged for 5 min at 450 g, and 3,860 µL of supernatant was removed carefully with a pipet. Samples were analyzed by Coulter CYTOMICS™ FC500 and XL-system II software (BeckmanCoulter) for 6 min each.

Statistical Analysis

Data are expressed as means ± standard deviation. Comparisons among groups were analyzed by ANOVA. Comparisons of categorical variables were carried out using Pearson’s χ²-test. Univariate analysis and multiple logistic regression analysis were carried out to investigate the relationship between CD34+ cell level and age, duration of diabetes, HbA1c, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, TG, estimated glomerular filtration rate, SBP and body mass index (BMI). Cumulative event-free survival was determined using univariate Kaplan–Meier analysis (log–rank test). The Cox’s proportional hazards ratio (HR) was used to estimate the relative risk of cardiovascular events adjusted for age, sex, smoking, history of cardiovascular diseases, dyslipidemia, hypertension, HbA1c level, BMI and statin use (reference was high tertile of CD34+). Hazard ratios and 95% confidence intervals (CI) are given. A value of P < 0.05 was considered statistically significant. All statistical analyses were carried out using JMP 8.0 (SAS Institute, Inc., Cary, NC, USA).

RESULTS

The median concentration of CD34+ cells in all patients was 0.91 ± 0.57 cells/µL. Age, duration of diabetes, BMI and TG were significantly associated with CD34+ cell level, whereas HbA1c, SBP, low-density cholesterol and glomerular filtration rate were not significantly associated with CD34+ cell level in univariate analysis (Table 1). Age was significantly associated with CD34+ cell level, adjusted by sex, HbA1c, SBP, duration of diabetes and smoking, (β = –0.229, P = 0.0311). In contrast, duration of diabetes, BMI and TG were not significantly associated with CD34+ cell level adjusted by age, sex, HbA1c, SBP and smoking. As shown in Table 2, the average CD34+ cell concentration of the low, middle and high tertile was 0.40 ± 0.12 cells/µL, 0.76 ± 0.12 cells/µL and 1.52 ± 0.45 cells/µL, respectively. We previously reported that patients of moyamoya disease showed high CD34+ cell levels (2.28 ± 0.53 cells/µL) compared with control subjects (0.89 ± 0.07 cells/µL)9. Compared with control subjects of this previous report, low and middle tertiles in the present study showed low CD34+ cell levels, whereas the high tertile showed high CD34+ cell levels. The age of the low tertile was significantly higher than that of
CD34+ cell are associated with CHD

Table 1 | Correlation to CD34+ cell number at baseline in univariate analysis

| Variable | r   | P     |
|----------|-----|-------|
| Age      | −0.293 | <0.001 |
| BMI      | 1.727 | 0.0172 |
| SBP      | 0.092 | 0.2148 |
| HbA1c    | −0.040 | 0.5917 |
| HDLC     | 0.013 | 0.8594 |
| HDLc     | −0.135 | 0.0612 |
| TG       | 0.2111 | 0.0032 |
| eGFR     | 0.0418 | 0.5643 |

BMI, body mass index; eGFR, estimated glomerular filtration rate; HbA1c, glycated hemoglobin; HDLC, high-density lipoprotein cholesterol; LDLC, low-density lipoprotein cholesterol; SBP, systolic blood pressure. Data are expressed to block e; BMI, body mass index; CVD, cardiovascular disease; DBP, diastolic blood pressure. The HbA1c level of the middle tertile was significantly higher than the high tertile. There was no significant difference of systemic blood pressure among the three tertiles. There was no significant difference of the rate of statin and angiotensin converting enzyme inhibitor/angiotensin receptor blocker use among the three groups (Table 2).

The average duration of follow up was 4.7 ± 2.1 years (range 2–9 years). During follow up, 49 of 192 patients experienced a CHD event and 14 patients experienced a CVD event. Kaplan–Meier analysis showed that patients in the low CD34+ tertile had significantly higher numbers of cardiovascular events compared with those in the high CD34+ tertile (P = 0.0052; Figure 1). In the Cox regression model adjusted for age, sex, dyslipidemia, hypertension, smoking, HbA1c, BMI, and statin and renin angiotensin system inhibitors use, patients in the low tertile of CD34+ cell concentration had a significantly higher incidence of CHD events compared with patients in the high CD34+ tertile, whereas the middle tertile of CD34+ cells had a tendency of a higher incidence of CHD, but it was not significantly compared with patients in the high CD34+ tertile (Table 3).

In the present study, age (HR 1.03, 95% CI 0.99–1.05, P = 0.946), HbA1c (HR 1.06, 95% CI 0.90–1.23, P = 0.946), BMI (HR 0.99, 95% CI 0.92–1.06, P = 1.06), presence of hypertension (HR 1.06, 95% CI 0.49–2.77, P = 0.895), current or past smoking (HR 1.20, 95% CI 0.68–2.16, P = 0.533) and use of angiotensin converting enzyme inhibitor/angiotensin receptor blocker (HR 0.98, 95% CI 0.56–1.74, P = 0.952) were not significantly associated with the incidence of CHD. Male sex tended to be associated with the incidence of CHD, but it was not significant (HR 1.50, 95% CI 0.82–2.88, P = 0.182). The presence of dyslipidemia (HR 3.64, 95% CI 1.13–22.27, P = 0.027), statin use (HR 2.07, 95% CI 1.14–3.96, P = 0.016) and history of cardiovascular diseases (HR 2.83, 95% CI 1.61–5.15, P = 0.0003) were significantly associated with the incidence of CHD. However, statin use was not significantly associated with the incidence of CHD adjusted by history of cardiovascular disease.

In contrast, although low levels of CD34+ cells tended to be associated with CVD, there was no significant difference in the incidence of CVD among the three tertiles (Table 3).

DISCUSSION

Previously, low CD34+ cell level was reported to be associated with cardiovascular event in patients with metabolic syndrome. Bielak et al. also reported that low CD34+ cell level was associated with coronary artery calcification in an elderly population without hypertension and diabetes. Thus, low CD34+ cell level is an important risk factor of cardiovascular disease in various populations. However, it was not clarified whether low CD34+ cell was a predictive factor of the incidence of cardiovascular disease. To the best of our knowledge, this is the first prospective study showing that low levels of CD34+ cells represent an independent risk factor for cardiovascular disease in Japanese patients with type 2 diabetes. In the present study, dyslipidemia and a history of cardiovascular disease were associated with the incidence of CHD, whereas other risk factors of CHD, such as

Table 2 | Baseline clinical characteristics of study participants according to the concentration of CD34+ cells

| Variable | High tertile (≥1.01) | Middle tertile (0.58–1.00) | Low tertile (≤0.57) |
|----------|---------------------|---------------------------|-----------------------|
| Age (years) | 61 ± 11 | 65 ± 10* | 67 ± 8** |
| Sex (male/female) | 45/20 | 41/24 | 39/23 |
| BMI | 26.6 ± 3.9 | 25.5 ± 4.0 | 25.0 ± 4.3* |
| SBP (mmHg) | 131 ± 14 | 131 ± 18 | 129 ± 15 |
| DBP (mmHg) | 73 ± 9 | 70 ± 10 | 71 ± 10 |
| HbA1c (%) | 8.8 ± 2.0 | 9.7 ± 1.8** | 9.1 ± 1.7 |
| Fasting blood glucose (mmol/L) | 9.32 ± 4.44 | 10.82 ± 4.72 | 8.77 ± 3.44 |
| Total cholesterol (mmol/L) | 5.18 ± 1.04 | 5.13 ± 1.14 | 4.95 ± 1.09 |
| Triglycerides (mmol/L) | 2.14 ± 1.33 | 1.93 ± 1.89 | 1.63 ± 0.87* |
| HDL cholesterol (mmol/L) | 1.11 ± 0.28 | 1.14 ± 0.34 | 1.19 ± 0.39 |
| Cr (µmol/L) | 840 ± 68.1 | 804 ± 49.5 | 840 ± 53.0 |
| CD34+ cell (cells/µL) | 1.52 ± 0.45 | 0.76 ± 0.12 | 0.40 ± 0.12 |
| Dyslipidemia (present/absent) | 57/8 | 54/11 | 55/7 |
| Hypertension (present/absent) | 60/5 | 57/8 | 50/12 |
| History of CHD and/or CVD (present/absent) | 27/38 | 33/32 | 34/28 |
| Retinopathy (present/absent) | 19/46 | 26/39 | 32/30 |
| Smoking (current/past/never) | 14/26/24 | 8/31/26 | 6/31/25 |
| Use of statins (yes/no) | 39/26 | 35/30 | 36/26 |
| Use of ACEI or ARB (yes/no) | 38/27 | 39/26 | 35/27 |

ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; BMI, body mass index; CVD, cerebrovascular disease; DBP, diastolic blood pressure; HbA1c, glycated hemoglobin; HDL, high-density lipoprotein; SBP, systolic blood pressure. Data are expressed means ± standard deviation. *P < 0.05, **P < 0.01 vs the highest CD34+ tertile.

the other two tertiles (Table 2). The BMI of the low tertile was significantly lower than the BMI of the high tertile (Table 2). The HbA1c level of the middle tertile was significantly higher
smoking, hypertension, HbA1c level, male sex and BMI, were not significantly associated with CHD event. The Japan Diabetes Complication Study (JDCS) showed that dyslipidemia and high HbA1c level were significantly associated with CHD events, whereas smoking, hypertension, male sex and BMI were not significant. Thus, the population of the present study did not show much difference in risk of CHD compared with the JDCS cohort except for HbA1c. These results suggest that simple and reproducible measurements of CD34+ cells can be used as a clinical marker of CHD progression, which might be suitable for screening a broad group of patients.

Previous studies have shown that low levels of circulating CD34+KDR+ cells, which are generally regarded as EPCs, predict cardiovascular events. Furthermore, patients with diabetes are reported to have lower numbers of CD34+KDR+ cells, suggesting that a decrease in the number of circulating EPCs is involved in the pathogenesis of diabetic macrovascular complications. However, in the present study, we measured levels of circulating CD34+ cells, but not CD34+KDR+ cells. CD34 is a marker of immature bone marrow-derived cells. One recent report showed that the level of circulating CD34+ cells is relatively low in young people, but it increases with age, especially in patients with cardiovascular risk factors, until a gradual decrease with the onset of a vascular event. Another recent report showed that levels of circulating CD34+ cells transiently increase after the onset of diabetes, although patients with diabetes have lower levels of CD34+ cells compared with individuals with normal glucose tolerance. Taken together, this suggests that bone marrow-derived cells participate in the maintenance of vascular homeostasis, and exhaustion of this population of cells causes cardiovascular disease.

In the present study, there was no significant association between circulating CD34+ cell number and HbA1c level, although duration of diabetes was significantly inversely associated with CD34+ cell number in univariate analysis. These results suggest that a period of high glucose exposure rather than the magnitude of hyperglycemia could contribute to the circulating CD34+ cell number. Indeed, a previous study reported that the CD34+ cell number of patients with ≥10 years’ duration of diabetes was lower than patients with duration <10 years.

In the present study, we prospectively showed for the first time that low levels of circulating CD34+ cells are significantly associated with CHD events in patients with diabetes. Intracoronary administration of CD34+ cell reduced the frequency of angina episodes and restored myocardial perfusion. Intramyocardial administration of CD34+ cells was also reported to reduce the frequency of angina episodes. Previously, we reported that the level of circulating CD34+ cells is inversely associated with plasma B-type natriuretic peptide levels in

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**Table 3** | Relative risk of cardiovascular event in patients with low CD34+ cell level adjusted by age, sex, smoking, history of cardiovascular diseases, dyslipidemia, hypertension, glycated hemoglobin level, body mass index, renin–angiotensin system inhibitor use and statin use

| Coronary heart disease | Cerebral vascular diseases |
|------------------------|---------------------------|
| HR 95% CI | P | HR 95% CI | P |
| Middle tertile | 1.91 | 0.98–3.81 | 0.058 | 1.61 | 0.39–8.09 | 0.515 |
| Low tertile | 2.61 | 1.22–5.96 | 0.013 | 1.04 | 0.24–5.38 | 0.96 |

CI, confidence interval; HR, hazard ratio. Reference is high tertile of CD34+ cell level.
patients with diabetes without apparent cardiovascular disease. Taken together, it is suggested that lack of circulating CD34+ cells could be involved in the progression of not only cardiac dysfunction, but also CHD in diabetes. Furthermore, a recent report showed that levels of CD34+, but not CD34+KDR+, cells are associated with cardiovascular events in patients with the metabolic syndrome. This report also showed that CD133, which is another marker of EPC, positive cell was not significantly associated cardiovascular event. Consistent with the present results, that report suggested that CD34+ cell counts might be a more useful clinical marker of cardiovascular disease progression in patients with diabetes than the number of EPCs.

Previously, we reported that CD34+ cells are involved in cerebral ischemia. Indeed, in cross-sectional analysis, we also reported that there was a significant association between CD34+ cell level and cerebral ischemia. However, unexpectedly, CVD events were not significantly associated with levels of CD34+ cells in the present study. Levels of CD34+ cells might be associated with asymptomatic cerebral infarction rather than symptomatic cerebral infarction, as CD34+ cells play a role in the cerebral microcirculation. Therefore, it is possible that we underestimated the incidence of CVD, as we defined CVD events as hospitalization for stroke in the present study. Indeed, the number of CVD events was lower compared with the number of CHD events in the present study.

Thus, low CD34+ cell level was significantly associated with CHD, but not CVD in the present study. These results suggest that progression of coronary artery atherosclerosis might have a different mechanism from cerebral arteries in diabetes. In fact, the risk factor of CHD was different from CVD in diabetic patients in the JDCS.

The present study had several limitations. First, the sample size of this study was relatively small. Second, it is possible that the number of events were underestimated, as we did not evaluate CHD and CVD in asymptomatic patients. Indeed, the number of CVD events in the present study might be too small to detect the association between CD34+ cell level and CVD event. Third, the influence of changes in the levels of CD34+ cells during the study period on cardiovascular events can scarcely be denied, as the concentration of CD34+ cells was measured only at baseline.

In conclusion, we have shown that decreased levels of circulating CD34+ cells are associated with CHD in patients with diabetes in a prospective study. The precise measurement of the concentration of circulating CD34+ cells could be a useful method for identifying patients with diabetes at elevated risk for CHD. Furthermore, it is suggested that treatments that increase CD34+ cell counts might be useful for the prevention of CVD in patients with diabetes.

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