Effects of Treatment for Diabetes Mellitus on Circulating Vascular Progenitor Cells

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Abstract. The circulating endothelial progenitor cells (EPCs) have an important role in angiogenesis, and the smooth muscle progenitor cells (SMPCs) participate in atherosclerosis. However, little is known about the effects of treatment of diabetes mellitus (DM) on EPCs and SMPCs. Therefore, we investigated the relations between the number of circulating vascular progenitor cells before and after the treatment for DM. Ten previously untreated DM patients were enrolled in this study. Blood samples were collected before and after treatment. The peripheral mononuclear cells were purified and cultured to differentiate them into EPCs and SMPCs. After two weeks, the number of EPCs was determined by Dil-labeled acetylated low density lipoprotein and lectin binding. The number of SMPCs was evaluated by immunocytochemical staining of \(\alpha\)-smooth muscle actin. Before treatment, the number of EPCs and SMPCs was significantly related to hemoglobin A1c and blood sugar. Serial examination revealed that improvement of glycemic control significantly increased the number of both EPCs and SMPCs. DM reduces the number of circulating EPCs and SMPCs according to its severity, and treatment of DM significantly increases the number of EPCs and SMPCs, which may be involved in angiogenesis and atherosclerosis in diabetes.

Keywords: diabetes mellitus, endothelial progenitor cell, smooth muscle progenitor cell, glycemic control

Introduction

Diabetes mellitus (DM) leads to progression of atherosclerosis and a poor outcome. DM, one of the coronary risk factors, is still a clinical challenge in multiple organ disorders, including vascular disease. Accumulating evidence shows that the lipid metabolic disorders in DM are important mechanisms of diabetic vascular disorder. In diabetic patients, lipoproteins may be altered by glycation. Glycation of low density lipoprotein causes its accumulation in the circulation and increased cholesteryl ester accumulation in macrophages. Glycation of high density lipoprotein increases cholesteryl ester accumulation in the arterial wall. Glycation also promotes oxidation of low density lipoprotein in patients with DM. DM may promote atherosclerosis and vascular injuries by these mechanisms (1). Hink et al. demonstrated that DM was associated with endothelial cell dysfunction (2). Recent studies have shown that circulating endothelial progenitor cells (EPCs) and smooth muscle progenitor cells (SMPCs), which are derived from bone marrow, can differentiate into vascular endothelial cells and vascular smooth muscle cells, thereby suggesting that EPCs have an important role in angiogenesis and that SMPCs participate in atherosclerosis (3, 4). Hill et al. showed that cardiovascular risk factors were correlated with the number of circulating EPCs (5). The severity of DM was inversely related to the number of circulating...
EPCs (6). The function of EPCs was impaired in an in vitro study. Vascular smooth muscle cells participate in vascular remodeling and in neointimal proliferation after coronary stent implantation and heart transplantation. Sata et al. demonstrated that bone marrow derived cells were involved in neointimal proliferation in a mouse model (7).

Although several studies have revealed a relationship between cardiovascular risk factors and the number of EPCs (5, 8), little is known about the influence of treatment of DM on circulating EPCs and SMPCs. The aim of this study is the serial evaluation of the number of EPCs and SMPCs before and after treatment of DM.

Material and Methods

Study population
Ten male diabetic patients who had not been treated with oral hypoglycemic agents or insulin were enrolled in this study. DM was defined as a blood sugar level (BS) greater than 126 mg/dl. Exclusion criteria included the acute coronary syndrome (acute myocardial infarction, unstable angina pectoris), congestive heart disease, malignancy, and infection. Before the treatment all the patients were given a retinal examination. Fresh blood was collected before and after treatment of DM. All blood samples were tested for white blood cell (WBC) count, BS, total cholesterol, and hemoglobin A1c (HbA1c). The patients were then treated with sulfonylurea, α-glucosidase inhibitor, pioglitazone, or insulin. No patients were administrated HMG-CoA inhibitors. The protocol was approved by the local ethics committee, and informed consent was obtained from all patients.

Mononuclear cell collection and vascular progenitor cell culture
Human mononuclear cells (MNCs) were isolated from 10 ml of peripheral blood by gradient centrifugation using the Lymphoprep tube (Axis-Shield, Oslo, Norway). After purification with 3 washing steps, $10^6$ MNCs were cultured on fibronectin-coated plates (4 wells). The MNCs were cultured in EBM-2 (Cambrex Bio Science Walkersville, Inc., Walkersville, MD, USA) with EGM-2 (Cambrex Bio Science Walkersville, Inc.) for 2 weeks to differentiate them into EPCs.

MNCs were also cultured in Hu Media (Kurabou, Osaka) with Hu MediaSG (Cambrex Bio Science Walkersville Inc.) supplemented with 15% fetal bovine serum (total of 20% fetal bovine serum), 10 ng/ml fibroblast growth factor, and 10 ng/ml platelet-derived growth factor for 2 weeks to differentiate them into SMPCs. The medium was changed every 4 days.

Characterization and proliferation assay of EPCs and SMPCs
After 2 weeks in culture, the EPCs were stained by 1,1'-dioctadecyl-3, 3', 3'-tetramethylindocarbocyanine perchlorate-acetylated low density lipoprotein (Dil-acLDL) (Molecular Probes Inc., Eugene, OR, USA) and FITC-lectin (Sigma, St. Louis, MO, USA). The SMPCs were stained by α-smooth muscle actin (α-SMA) (Sigma) and an alkaline phosphatase substrate kit I (Vecter Laboratories Inc., Burlingame, CA, USA) by an immunochemical staining method. The acLDL and lectin double positive cells were defined as EPCs; α-SMA positive cells were defined as SMPCs. The EPCs and SMPCs were quantified by random microscopic fields (×200, ×400, respectively) (Fig. 1). The methods of the evaluation of EPCs and SMPCs numbers were described in detail previously (6, 9, 10).

Statistical analyses
All analyses were performed with Statview (SAS Institute Inc., Cary, NC, USA). Values were expressed as the mean ± S.D. and tested by two-tailed Student’s $t$-tests and the Dunnett post-hoc test. Values of $P<0.05$ in both two-tailed Student’s $t$-tests and the Dunnett post-hoc test were considered to be statistically significant. $P$ values of the two-tailed Student’s $t$-tests are shown in the text.

Results

Patient characteristics and follow-up
We cultured the blood samples provided by the 10 patients before treatment. All patients were male, and their mean age was 56.1 ± 5.6 years (range 46 – 66). The mean values of WBC, total cholesterol, BS, and HbA1c were 5610 ± 936/µl, 194.2 ± 46.5 mg/dl, 246.0 ± 51.1 mg/dl, and 10.1 ± 2.4%, respectively. No patients were obese (obesity was defined as a body mass index greater than 25.0). After treatment, blood samples from 8 patients were collected, 2 patients dropped out, terminating treatment at their own discretion. The characteristics of the 8 patients are summarized in Table 1. The follow-up period was 34.1 ± 11.5 days.

The number of EPCs and SMPCs before treatment
The mononuclear cells from 10 pre-treatment blood samples were cultured for EPCs and SMPCs. The number of EPCs in patients with untreated DM was 18.2 ± 8.5 cells/high powered field (HPF). Figure 2, a and b, show that the number of EPCs had a strong inverse correlation with BS ($r = -0.65$, $P = 0.04$) and
HbA1c ($r = -0.73$, $P = 0.01$). However, there was no correlation between the number of EPCs and age, WBC, or total cholesterol. The number of SMPCs in patients with untreated DM was $13.3 \pm 6.1$ cells/HPF. Figure 2, c and d, show that there was a significant correlation between the number of SMPCs and BS ($r = -0.63$, $P = 0.04$) and between the number of SMPCs and HbA1c ($r = -0.73$, $P = 0.01$). There was no significant correlation between the number of SMPCs and age, WBC, or total cholesterol. Before treatment of DM, the number of EPCs was not significantly related to the number of SMPCs ($r = 0.51$, $P = 0.13$).

**The relations between the number of EPCs and SMPCs and diabetic retinopathy**

As shown in Fig. 3, the 10 patients before the treatment (including the 2 patients that dropped out) were divided into two groups: retinopathy group ($n = 4$)
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and non-retinopathy group (n = 6) by retinal examination. The stage of diabetic retinopathy of all patients in retinopathy group was non-proliferative retinopathy. BS in the retinopathy group was significantly higher than in non-retinopathy group (284 ± 57 mg/dl in retinopathy group versus 221 ± 28 mg/dl, P = 0.04). HbA1c in the retinopathy group was also significantly higher than in the non-retinopathy group (12.4 ± 1.9% in retinopathy group vs 8.6 ± 1.1%, P = 0.004). The number of EPCs in the retinopathy group was significantly smaller than in non-retinopathy group.

Fig. 2. The relationship between the number of progenitor cells and hemoglobin A1c or blood sugar. a: The relationship between the number of EPCs and BS, b: the relationship between the number of EPCs and HbA1c, c: the relationship between the number of SMPCs and BS, d: the relationship between the number of SMPCs and HbA1c. BS: blood sugar, EPC: endothelial progenitor cell, HbA1c: hemoglobin A1c, SMPC: smooth muscle progenitor cell, HPF: high powered field.

Fig. 3. The comparison of the number of EPCs and SMPCs between the DM patients with and without retinopathy. These graphs demonstrated the number of EPCs (left) and SMPCs (right). Solid bars are the retinopathy group. Open bars are the non-retinopathy group.
the non-retinopathy group (10.5 ± 4.5 cells/HPF in retinopathy group vs 23.3 ± 6.2 cells/HPF, \( P = 0.008 \)). The number of SMPCs in the retinopathy group was about half of the number of SMPCs in the non-retinopathy group. However, there was no significant difference between the two groups (9.3 ± 6.2 cells/HPF in retinopathy group vs 16.0 ± 4.7 cells/HPF, \( P = 0.09 \)).

**Discussion**

Accumulating evidence shows that EPCs have an important role in angiogenesis. Recent studies have investigated circulating EPCs in patients with cardiovascular disease (6, 11, 12). EPCs increased in patients with acute myocardial infarction and reached a peak at 7 days after the onset. Coronary risk factors (Framingham risk score) were inversely correlated with the number of EPCs (5, 8). DM reduced the number of circulating EPCs and impaired their function. The functional characteristics of EPCs included the number of EPCs, tube formation, adhesion, and angiogenic growth factors secreted from EPCs (for example, vascular endothelial growth factor, hepatocyte growth factor, and granulocyte-stimulating factor) (12 – 14). However, the clinical effects of treatment of DM on circulating EPCs remain unclear.

Simper et al. showed that vascular smooth muscle cells may originate from bone marrow derived cells: “smooth muscle progenitor cells” (SMPCs). Sata et al. demonstrated that bone marrow derived cells took part in neointimal and vascular smooth muscle cell formation.

**Fig. 4.** The effects of treatment of DM on laboratory results and the number of EPCs and SMPCs. a: BS, b: HbA1c, c: the number of EPCs, d: the number of SMPCs. DM: diabetes mellitus, EPC: endothelial progenitor cell, BS: blood sugar, HbA1c: hemoglobin A1c, SMPC: smooth muscle progenitor cell, HPF: high powered field, f/u: follow up.

Table 1 lists patient characteristics and laboratory results before and after treatment. All patients were successfully treated by insulin injection, sulfonylurea, and/or rosiglitazone. Table 1 and Fig. 4a show that treatment of DM significantly improved BS from 250.3 ± 57.0 to 104.8 ± 14.9 mg/dl (\( P = 0.01 \)). Table 1 and Fig. 4b show that total cholesterol and HbA1c level were not significantly changed (total cholesterol: from 197.3 ± 51.7 to 189.9 ± 42.7 mg/dl, \( P = 0.78 \); HbA1c: from 10.3 ± 2.6% to 8.9 ± 1.4%, \( P = 0.12 \)). Figure 4c shows that the number of EPCs was significantly increased after treatment of DM (from 18.0 ± 9.0 to 27.1 ± 11.0 cells/HPF (×200), \( P = 0.03 \)). Figure 4d shows that the number of SMPCs was also significantly increased after treatment of DM (from 12.8 ± 6.6 to 22.3 ± 10.8 cells/HPF (×400), \( P = 0.04 \)).
after vascular injury in a mouse model (7). Vascular smooth muscle cells are closely related with atherosclerosis. Therefore, SMPCs are thought to be essential for atherosclerosis (15). Although both EPCs and SMPCs originate from peripheral MNCs, it is still unclear why EPCs and SMPCs have different effects on vascular structure. Against this background, we evaluated the number of EPCs and SMPCs in patients with DM.

Previous studies have demonstrated an inverse correlation between the number of EPCs and HbA1c. The present study was consistent with previous studies, in that the number of cultured EPCs before treatment significantly correlated with HbA1c ($r = -0.73$, $P = 0.01$). In the present study, the number of EPCs and SMPCs was significantly correlated with pre-treatment HbA1c and BS. Because BS reflects HbA1c levels, it is not surprising that there were strong correlations between BS and the number of EPCs and SMPCs. Our results therefore suggested that the number of EPCs and SMPCs may be a useful indicator of the severity of DM.

Although the number of EPCs had no correlation with the number of SMPCs, serial evaluation revealed that the number of EPCs and SMPCs increased significantly in response to improvement in BS. BS after treatment was significantly improved compared with the pretreatment level ($P = 0.01$). When diabetic glycemic control is poor, the proliferation of EPCs and SMPCs may be impaired (12). Therefore, the number of EPCs and SMPCs increased significantly after adequate treatment. In the present study, HbA1c was not significantly reduced when compared with pretreatment levels ($P = 0.12$). The discrepancy between BS and HbA1c reflected the length of the follow-up period. Treatment usually takes several months to affect HbA1c levels. Because the follow-up period in the present study was 34.1 ± 11.5 days, the mean HbA1c decreased, but not significantly. Interestingly, the number of EPCs and SMPCs improved earlier than the improvement in HbA1c level. The number of circulating EPCs and SMPCs may be more sensitive to diabetic control than to HbA1c levels.

SMPCs are known to participate in atherosclerosis. Accumulation of smooth muscle cells plays an important role in atherosclerosis and neointimal formation. However, we showed that improvement of glycemic control increased the number of SMPCs. SMPCs may be one of several causes of progression of atherosclerosis and similarly, EPCs in angiogenesis. The roles of EPCs and SMPCs in vascular disorders are in sharp contrast (3, 4, 7). In the present study, the number of SMPCs and EPCs was increased by treatment of DM. Because both EPCs and SMPCs originate from human peripheral MNCs, EPCs, and SMPCs may have the same characteristics of mobilization to circulating blood. The culture environment may affect the differentiation from MNCs into EPCs and/or SMPCs. Otherwise, because hyperglycemia leads to vascular damage, migration and adhesion of EPCs and SMPCs to injured vessels may result in decreased cell numbers by consumption of circulating EPCs and SMPCs (16). In this study, both the number of EPCs and SMPCs in non-retinopathy patients was larger than in retinopathy patients. This hypothesis may explain why improvement of glycemic control increased the number of EPCs and SMPCs.

There were several limitations in this study. Firstly, the number of patients was small. Secondly, the treatment of DM differed between patients. Thirdly, we evaluated only the numbers of vascular progenitor cells, not their function.

In conclusion, we demonstrated that the number of EPCs and SMPCs was increased by treatment of DM. Because human peripheral vascular progenitor cells are involved in angiogenesis and atherosclerosis, further functional investigation of vascular progenitor cells is needed to clarify the mechanism of vascular disorders.

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References


