Endothelial Progenitor Cell Dysfunction

A Novel Concept in the Pathogenesis of Vascular Complications of Type 1 Diabetes

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Type 1 diabetes is associated with reduced vascular repair, as indicated by impaired wound healing and reduced collateral formation in ischemia. Recently, endothelial progenitor cells (EPCs) have been identified as important regulators of these processes. We therefore explored the concept that EPCs are dysfunctional in diabetes. The number of EPCs obtained from type 1 diabetic patients in culture was 44% lower compared with age- and sex-matched control subjects ($P < 0.001$). This reduction was inversely related to levels of HbA_{1c} ($R = -0.68$, $P = 0.01$). In addition, we demonstrated that patient EPCs were also impaired in function using an in vitro angiogenesis assay. Conditioned media from patient EPCs were significantly reduced in their capacity to support endothelial tube formation in comparison to control EPCs. Therefore, despite culturing the EPCs under normoglycemic conditions, functional differences between patient and control EPCs were maintained. Our findings demonstrate that adverse metabolic stress factors in type 1 diabetes are associated with reduced EPC numbers and angiogenicity. We hypothesize that EPC dysfunction contributes to the pathogenesis of vascular complications in type 1 diabetes. *Diabetes* 53: 195–199, 2004

Hyperglycemia is associated with endothelial cell (EC) dysfunction and reduced neovascularization in response to tissue ischemia, processes that are essential for wound healing and prevention of cardiovascular ischemia (1–3). A growing body of evidence indicates that neovascularization does not exclusively rely on proliferation of local ECs but also involves bone marrow–derived circulating stem cells (4). These cells can be cultured from the circulating mononuclear cell (MNC) fraction and are commonly referred to as endothelial progenitor cells (EPCs) because they exhibit characteristic endothelial surface markers and properties. Moreover, a number of studies have shown that injected EPCs home to sites of ischemia, incorporate into the newly formed capillaries, and augment neovascularization (5). Consequently, if EPCs are critical to endothelial maintenance and repair, EPC dysfunction could contribute to the pathogenesis of ischemic vascular disease. Indeed, studies have demonstrated that, in patients with cardiovascular risk factors, the number of EPCs that can be isolated from peripheral blood is reduced (6) and EPC function is impaired (7,8). It was recently reported that a strong inverse correlation exists between the number of EPCs and the subjects' combined Framingham risk factor score (9). In addition, measurements of flow-mediated brachial-artery reactivity also revealed a significant relation between endothelial function and the number of EPCs, supporting a role for EPCs in the maintenance of endothelial integrity.

In this study, we investigated the hypothesis that EPC dysfunction exists in type 1 diabetic patients. To that end, we determined the number of EPCs obtained from peripheral blood of type 1 diabetic patients and its relation to glycemic control. Furthermore, we compared the capacity of patient and control EPCs to support endothelial tube formation in vitro.

RESEARCH DESIGN AND METHODS

After informed consent was obtained, peripheral blood samples were collected from 20 type 1 diabetic patients and 20 age- and sex-matched healthy control subjects. Patients with type 1 diabetes, diagnosed at least 1 year before entering the study, were recruited from the Department of Diabetology of the University Medical Center Utrecht. All patients were treated with insulin for at least 1 year. Patients with manifest macrovascular disease were excluded. Other exclusion criteria included smoking, alcohol abuse, liver disease, creatinine >120 μmol/l, and untreated thyroid disease. If patients were treated with vasoactive medication (angiotensin-converting enzyme inhibitors, statins, aspirin, nonsteroidal anti-inflammatory drugs, angiotensin II antagonists, folic acid, or vitamins), treatment was stopped at least 3 weeks before blood withdrawal. Whole blood was used for assessment of HbA_{1c} using an immunochemical method (Tina-quant; Roche/Hitache, Mannheim, Germany). Glucose concentrations were measured by a glucose oxidase technique. The study protocol was approved by the Ethics Committee of the University Medical Center Utrecht.

EPC isolation and characterization. Peripheral blood was obtained in blood collection tubes containing EDTA (Venoject; Terumo Europe N.V., Leuven, Belgium). EPCs were cultured as described (10). Briefly, MNC

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Received for publication 17 July 2003 and accepted in revised form 25 September 2003.

EC, endothelial cell; EPC, endothelial progenitor cell; FITC, fluorescein isothiocyanate; HUVEC, human umbilical vein EC; MNC, mononuclear cell; PB, peripheral blood; UEA, *Ulex europaeus agglutinin*.

TABLE 1
Subject characteristics

	Control subjects	Type 1 diabetic patients
n	20	20
Age (years)	39.9 ± 13.9	40.7 ± 14.8
Sex (M/F)	13/7	13/7
BMI (kg/m^2)	23.03 ± 2.6	24.7 ± 1.9
Glucose (mmol/l)	5.2 ± 1.3	7.8 ± 3.5
HbA_{1c} (%)	—	8.3 ± 1.5
Duration of diabetes (years)	—	21.1 ± 15.2

Data are means ± SD.

fractions were isolated from 60 ml whole blood by density gradient centrifugation (Histopaque 1077; Sigma, St. Louis, MO). MNCs were plated at a density of 1×10^6 cells per cm^2 on six-well culture plates coated with 2% gelatin (Sigma) in M199 medium supplemented with 20% FBS (Invitrogen; Breda, the Netherlands), 0.05 mg/ml Bovine Pituitary Extract (Invitrogen), antibiotics, and 10 units/ml heparin (Leo Pharma BV, Breda, the Netherlands). After 4 and 7 days of culture, EPC characteristics were confirmed on the basis of morphology and by fluorescent confocal immunohistochemistry using *Ulex europaeus agglutinin* (UEA)-1 (Vector, Burlingame, CA), a CD31 antibody (Dako, Glostrup, Denmark), and DiI-labeled acetylated LDL (Molecular Probes, Leiden, the Netherlands).

Flow cytometry analyses. Quantitative determination of the percentage of cells undergoing apoptosis was determined using an Annexin V apoptosis detection kit (BD Biosciences Pharmingen, San Diego, CA) according to the manufacturer's protocol. Briefly, fresh MNCs and EPCs, cultured for 4 days, were isolated as described. After the recommended washing steps, 1×10^6 cells were incubated for 15 min with fluorescein isothiocyanate (FITC)-conjugated Annexin V in binding buffer (BD Biosciences) in the dark. Annexin binding was measured by flow cytometry (FACScan, BD Biosciences) within 1 h and quantified using CellQuest software (BD Biosciences). Mean levels of Annexin V binding were determined using specific monocyte and lymphocyte gates in the forward-sideward scatter plots and expressed as arbitrary units of fluorescence.

In vitro angiogenesis assay. The angiogenic activity of EPC-conditioned media was assessed using an in vitro angiogenesis assay kit (Chemicon, Temecula, CA) and passage 2 primary human umbilical vein ECs (HUVECs). Conditioned media were obtained by replacing the medium of 4-day EPC cultures with serum-free EC basal medium-2 (Clonetics, Baltimore, MD) supplemented with EGM-2 single aliquots (growth factors like vascular endothelial growth factor and basic fibroblast growth factors were omitted) and cultured for an additional 30 h. EPCs were counted, and in subsequent experiments conditioned media were diluted to correct for EPC numbers. After 16 h, tube formation by HUVECs was measured by staining the viable cells with Calcein-AM (5 μg/ml) (Molecular Probes). Total tube area was determined using images obtained with an inverted fluorescence microscope and the Scion Imaging software (Scion Corporation, Frederick, MD) and expressed in arbitrary units.

Statistical analysis. Statistical analysis was performed with a Student's *t* test, and results are expressed as mean ± SD. Linear regression analyses and Pearson correlation were used for comparison of the number of EPCs and HbA_{1c}. Probability values of $P < 0.05$ were considered statistically significant.

RESULTS

Patient characteristics. Subject characteristics are presented in Table 1. The group of diabetic patients is representative of a type 1 diabetic population without macrovascular complications. Patients widely ranged in age and HbA_{1c}. Background, preproliferative, and proliferative retinopathy was present in four, three, and two patients, respectively. Microalbuminuria was present in 4 of 20 patients.

Correlation of reduced EPC number and HbA_{1c} in type 1 diabetic patients. Peripheral blood MNCs from type 1 diabetic patients and control subjects were cultured and differentiated. From 4 days on, next to cell clusters, EPCs appeared with a typical spindle-shaped morphology (4). EPCs were further characterized by assessing the uptake of DiI-labeled acLDL, the binding of the lectin UEA-1, and the presence of the CD31 antigen, all three characteristic features of cells in the endothelial lineage. At day 4, 80% of the attached cells already stained positive for all three markers. At day 7, the spindle-shaped morphology appeared more pronounced and >90% of the cells stained positive for all three markers. Figure 1*A* shows a representative picture of a dual staining of a 7-day EPC culture, and Fig. 1*B* represents a confocal image of a representative spindle-shaped cell in a 7-day EPC culture that shows uptake of DiI-labeled acLDL (red) and membrane staining with an anti-CD31 antibody (green).

After the phenotypic characterization of our EPC cultures, we assessed the number of EPCs that could be obtained from 4-day cultures derived from the peripheral blood MNC fraction of type 1 diabetic patients and age- and sex-matched control subjects. We observed a significant 44% decrease in the number of EPCs obtained from 60 ml peripheral blood of the type 1 diabetic patients compared with the nondiabetic control subjects ($4.3 \pm 2.3 \times 10^6$ vs. $7.8 \pm 3.3 \times 10^6$, $P < 0.001$, Fig. 2*A*). Moreover, linear regression analyses revealed an inverse relationship between the number of EPCs and the HbA_{1c} in the patients ($R = -0.68$, $P = 0.01$, Fig. 2*B*).

No evidence for increased apoptosis in cultured EPCs from type 1 diabetic patients. To evaluate if the reduction in the number of EPCs was due to apoptosis, we analyzed the binding of Annexin V to phosphatidylserine in both EPCs cultured for 4 days and the MNC fraction they originated from. The display of phosphatidylserine in the outer leaflet of the plasma membrane of cells is considered an early marker of apoptosis. Quantitative analyses of data

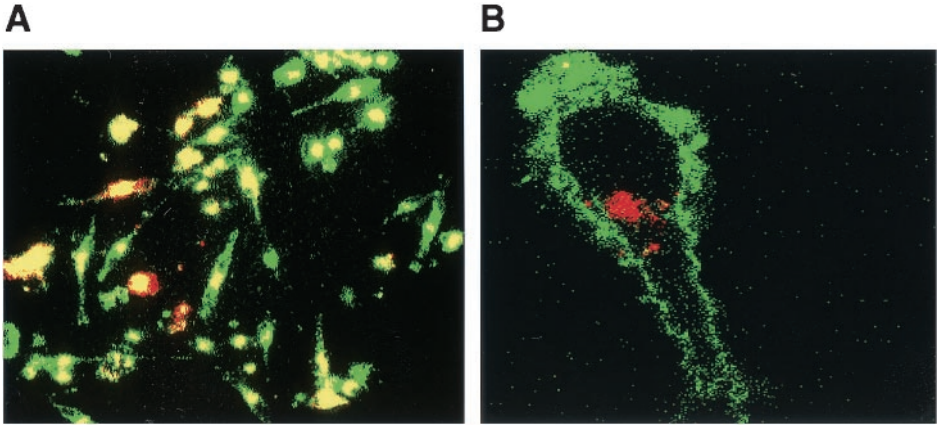

FIG. 1. EPC characterization. *A*: Fluorescent microscopy shows a representative EPC culture containing >90% cells with a spindle-shaped morphology both staining positive for FITC-labeled Ulex europaeus agglutinin and DiI-labeled acetylated LDL. *B*: Confocal microscopy picture showing dual staining of EPCs by CD31 antibodies and uptake DiI-labeled acLDL.

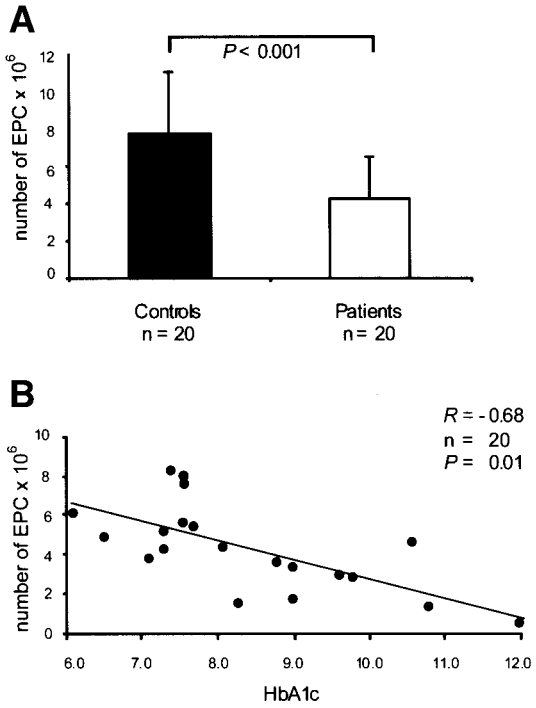

FIG. 2. MNCs were isolated out of 60 ml peripheral blood from 20 control subjects and 20 type 1 diabetic patients. MNCs were plated on gelatin-coated dishes, and EPCs were cultured for 4 days. *A*: The total EPC numbers were counted and a 44% reduction was seen in the type 1 diabetic patients compared with the healthy control subjects. *B*: This reduction in EPC numbers inversely correlated with glycemic control assessed by HbA_{1c}.

obtained by flow cytometry revealed no significant difference in the mean levels of Annexin V binding to EPCs cultured from patients or control subjects when gated for viable cells (22.3 ± 10.7 and 20.4 ± 9.2, respectively, $P = 0.54$), suggesting no increase in early apoptosis in these cells. Likewise, in fresh MNC fractions, which were the source of our EPC cultures, we also did not observe a significant increase in the mean levels of Annexin V binding of the total MNC fraction in the patient group compared with that of the control subjects (370.9 ± 78.5 vs. 304.7 ± 139.5, $P = 0.17$).

EPCs from patients with type 1 diabetes are impaired in their potential to augment angiogenesis in vitro. EPCs are thought to augment neovascularization not only by integration of these cells into newly developing capillaries, but also in a paracrine fashion through the secretion of angiogenic growth factors (5). To investigate whether this paracrine function is affected in type 1 diabetes, we determined the angiogenic potential of EPC (day 4)-conditioned medium in an in vitro angiogenesis model. In this angiogenesis model, the degree of tube formation of mature ECs on a solid gel of matrix proteins can be evaluated. As tube formation in this assay is dependent on the presence of angiogenic stimuli we assessed the angiogenic capacity of cultured EPC. HUVECs were seeded on extracellular matrix and subjected overnight to conditioned medium of diabetic EPCs ($n = 10$) or of EPCs from age-matched control subjects ($n = 10$). Conditioned media of healthy control subjects markedly stimulated tube formation when compared with nonconditioned media ($EGM^{-/-}$), demonstrating that EPCs can facilitate angiogenesis in a paracrine fashion (Fig. 3). In contrast, the conditioned media of the patient EPCs significantly reduced tube formation when compared with nonconditioned media, suggesting that cultured EPCs from type 1 diabetic patients can secrete factors that impair angiogenesis in vitro.

DISCUSSION

Our data support the hypothesis that EPCs are dysfunctional in patients with type 1 diabetes. First, a reduced number of EPCs could be cultured from the MNC fraction of type 1 diabetic patients when compared with healthy control subjects. A reduction of the number of EPCs in patients with risk factors for coronary artery disease has also been reported by others (6–8). Our data are consistent with a previous report by Schatteman et al. (7), who showed that $CD34^+$ cells derived from type 1 diabetic patients produced less differentiated ECs than their non–diabetic derived counterparts. Here, we have extended their observation by showing that in type 1 diabetes, EPC numbers inversely correlate with HbA_{1c} levels, demon-

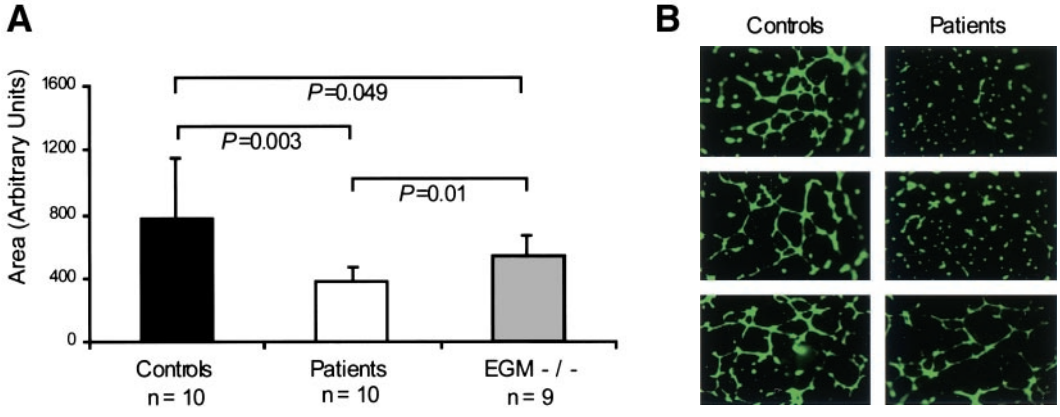

FIG. 3. Angiogenic capacity of conditioned media of EPCs in an in vitro angiogenesis assay. HUVECs were plated on extracellular matrix and subjected to EPC-conditioned media. Tubule networks were formed overnight and structures were visualized with Calcein-AM staining. Quantitative analyses of tube formation were performed using an imaging program. *A*: A significant inhibition of tube formation of patient EPC conditioned media is shown. *B*: Representative pictures of the stained tubule networks formed after incubation of HUVECs with the conditioned media of EPCs derived from thee different patients and their age- and sex-matched control subjects.

strating that the degree of glycemic dysregulation directly affects EPC proliferation or differentiation.

As the number of circulating leukocytes is tightly regulated by the balance between proliferation and apoptosis (11), and as increased apoptosis has been associated with the adverse metabolic state and oxidative stress in diabetes (12), we hypothesized that increased apoptosis could explain the reduced EPC numbers. When we analyzed early apoptosis in EPCs cultured for 4 days or in the freshly isolated MNC fraction they originate from, no significant difference in the mean levels of Annexin V binding was detected in the type 1 diabetic patient group compared with the control group. These data suggest that increased apoptosis is unlikely to be involved in the reduction of the number of EPCs in type 1 diabetic patients. An alternative explanation for the lower EPC counts in this study could be that the EPC precursors in the MNC fraction have an impaired capacity to adhere and/or differentiate in our culture conditions. This would represent yet another level of EPC dysfunction that is currently under investigation.

Irrespective of the underlying mechanism, a reduced number of EPCs is likely to impact on vascular integrity as it was recently reported that, in healthy men, the number of EPCs serve as a surrogate marker for vascular function and cumulative cardiovascular risk (9).

Having established that the number of EPCs is reduced in type 1 diabetes, we determined if the function of the remaining cells was altered compared with the control subjects. So far, only few studies addressed the subject of EPC dysfunction. It was reported that EPCs isolated from patients with coronary artery disease displayed an impaired migratory response (6) and that, in type 2 diabetes, EPC adhesion to stimulated ECs is impaired (8). Although EPCs enhance new vessel formation, these cells do not form the entire vessel de novo and the process always includes mature ECs (13). Recently, it has been suggested that a major function of EPCs could be the secretion of angiogenic factors to activate resident mature ECs (14). Here we show that the angiogenic capacity of conditioned media from patient-derived EPCs is not only reduced, but it also may contain an inhibitor for in vitro tube formation of ECs.

Furthermore, our data demonstrate that type 1 diabetes is associated with altered EPC function and that these changes are observed even though the EPCs were cultured for 4 days in a normoglycemic environment. To begin to investigate whether these functional changes are reflected in the gene expression profiles of the diabetic EPCs, we have performed preliminary Affymetrix DNA microarray analyses. Indeed, we observed extensive differential gene expression in patient-derived cultured EPCs compared with control subjects. Interestingly, many of these alterations have been reported to be associated with diabetes in general, hyperglycemia, or oxidative stress such as plasminogen activator inhibitor 1 (15) and osteopontin (16). This demonstrates that EPCs may function as "bio-sensors," translating metabolic cues into altered gene expression. How the EPCs "remember" its metabolic descent in culture is unknown and needs further investigation.

Taken together, we demonstrate that EPCs cultured from type 1 diabetic patients are reduced in number and function. As a consequence, EPC dysfunction may reduce the vascular regenerative potential of this patient group and thereby contribute to the pathogenesis of vascular complications in type 1 diabetes.

Finally, the notion that EPC dysfunction exists in certain patient categories, such as type 1 diabetes, may have implications for currently explored cell-based clinical strategies to enhance tissue perfusion in patients with ischemic coronary and peripheral artery disease (17,18). EPCs or MNCs isolated from these patients for autologous cell transplantation may retain their dysfunctional characteristics in vivo and as a consequence display a reduced capacity to augment therapeutic neovascularization. Therefore, it could be useful to set progenitor quality criteria and perform EPC function tests (such as assays for angiogenic growth factor secretion, adhesion, or migration) in order to obtain optimal cells for transplantation.

ACKNOWLEDGMENTS

Supported by the Netherlands Heart Foundation, The Hague, by Grants 2000.019 and 2002B157. B.B. is supported by a fellowship of the Dutch Academy of Arts and Sciences. M.C.V. is supported by the Netherlands Organization for Scientific Research (NWO Grant 016.036.041). F.J.T.S. is supported by the Dutch Academy of Arts and Sciences, the Bekales Foundation, and the Anna and Maurits de Cock Foundation.

We are grateful to Dr. T. Murohara from the Nagoya University Graduate School of Medicine for teaching us how to culture EPCs.

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