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Chapter 3

Endothelial Progenitor Cell Dysfunction: a Novel Concept in the Pathogenesis of Vascular Complications of Type 1 Diabetes

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Abstract

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 (EPC) have been identified as important regulators of these processes. We therefore explored the concept that EPC are dysfunctional in Diabetes Mellitus. The number of EPC obtained from type 1 Diabetes patients in culture was 44% lower compared to age- and gender- matched controls ($P < 0.001$). This reduction was inversely related to levels of HbA1c ($R = -0.68$, $P = 0.01$). In addition, we demonstrated that patient EPC were also impaired in function using an *in vitro* angiogenesis assay. Conditioned media from patient EPC were significantly reduced in their capacity to support endothelial tube formation in comparison to control EPC. Therefore, despite culturing the EPC under normoglycemic conditions, functional differences between patient and control EPC 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.

Introduction

Hyperglycemia is associated with endothelial cell dysfunction and reduced neovascularization in response to tissue ischemia, processes that are essential for wound healing and prevention of cardiovascular ischemia¹⁻³. A growing body of evidence indicates that neovascularization does not exclusively rely on proliferation of local endothelial cells but also involves bone marrow-derived circulating stem cells⁴. These cells can be cultured from the circulating mononuclear cell fraction and are commonly referred to as endothelial progenitor cells (EPC) because they exhibit characteristic endothelial surface markers and properties. Moreover, a number of studies have shown that injected EPC home to sites of ischemia, incorporate into the newly formed capillaries and augment neovascularization⁵. Consequently, if EPC 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 EPC that can be isolated from peripheral blood is reduced⁶ and EPC function is impaired^{7,8}. It was recently reported that a strong inverse correlation exists between the number of EPC and the subjects' combined Framingham risk factor score⁹. In addition, measurements of flow-mediated brachial-artery reactivity also revealed a significant relation between endothelial function and the number of EPC, supporting a role for EPC in the maintenance of endothelial integrity.

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

Research design and methods

Patient characteristics

After informed consent was obtained, peripheral blood (PB) samples were collected from twenty type I diabetes patients and twenty age- and gender-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 one year. Patients with manifest macrovascular disease were excluded. Other exclusion criteria included: smoking, alcohol abuse, liver disease, creatinine >120 $\mu\text{mol/l}$ and untreated thyroid disease. If patients were treated with vasoactive medication (angiotensin-converting enzyme inhibitors, statins, aspirin, non-steroidal 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 HbA1c 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). EPC were cultured as described¹⁰. Briefly, mononuclear cell fractions (MNC) were isolated from 60 ml whole blood by density gradient centrifugation (Histopaque 1077, Sigma, St. Louis, MO). MNC were plated at a density of 1×10^6 cells per cm^2 on 6-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 U/ml heparin (Leo Pharma BV, Breda, The Netherlands). After four and seven days of culture, EPC characteristics were confirmed on the basis of morphology and by fluorescent confocal immunohistochemistry using Ulex europaeus agglutinin (UEA)-I (Vector, Burlingame, CA, USA), a CD31 antibody (DAKO Diagnostics, 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, USA) according to the manufacturers protocol. Briefly, fresh MNC and EPC, cultured for four 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, USA) and passage two primary human umbilical vein endothelial cells (HUVEC). Conditioned media were obtained by replacing the medium of 4 day EPC cultures with serum-free endothelial cell basal medium-2 (Clonetics, Baltimore MD, USA) supplemented with EGM-2 single aliquots (growth factors like VEGF and bFGF were omitted) and cultured for an additional 30 h. EPC were counted and in subsequent experiments conditioned media were diluted to correct for EPC numbers. After 16 h, tube formation by HUVEC was measured by staining the viable cells with Calcein-AM (5µg/ml) (Molecular Probes). Total tube area was determined using images obtained with an inverted fluorescence microscope and the Scion Imaging software (Scion Corporation, Maryland, USA) and expressed in arbitrary units.

Statistical Analysis

Statistical analysis was performed with a Student's *t* test, and results are expressed as mean $\pm$ SD. Linear regression analyses and Pearson correlation were used for comparison of the number of EPC and glycosylated hemoglobin (HbA1c). 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 diabetes population without macrovascular complications. They had a wide range of age and HbA1c. Background, preproliferative and proliferative retinopathy was present in 4, 3 and 2 patients, respectively. Microalbuminuria was present in 4 out of 20 patients.

Table 1: Subject characteristics

	Controls	Type 1 diabetes 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)	23.0 ± 2.6	24.7 ± 1.9
Glucose (mmol/l)	5.2 ± 1.3	7.8 ± 3.5
HbA1c (%)	-	8.3 ± 1.5
Duration of diabetes (years)	-	21.1 ± 15.2

Values are mean ± SD.

Correlation of reduced EPC number and glycosylated hemoglobin in type 1 diabetes patients

Peripheral blood MNC from type 1 diabetes patients and controls were cultured and differentiated. From four days on, next to cell clusters, EPC appeared with a typical spindle-shaped morphology⁴. EPC 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. Already at day four, 80% of the attached cells stain positive for all three markers. At day seven the spindle shape morphology appears more pronounced and over 90% of the cells stain positive for all three markers. Figure 1A, shows a representative picture of a dual staining of a seven day EPC culture and Figure 1B, represents a confocal image of a representative spindle shaped cell in a seven day EPC culture, that shows uptake of DiI-labeled acLDL (red) and membrane staining with an anti-CD31 antibody (green).

Following the phenotypic characterization of our EPC cultures we assessed the number of EPC that could be obtained from four day cultures derived from the peripheral blood MNC fraction of type 1 diabetes patients and age- and gender-matched controls. We observed a significant 44% decrease in the number of EPC obtained from 60 ml peripheral blood of the

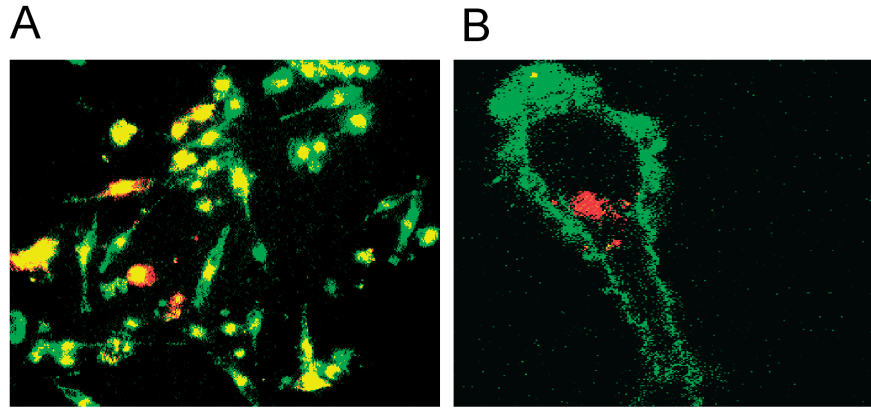


Figure 1.

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

type 1 diabetes patients compared to the non-diabetic controls ($4.3 \pm 2.3 \times 10^6$ vs. $7.8 \pm 3.3 \times 10^6$, $P < 0.001$, Fig. 2A). Moreover, linear regression analyses revealed an inverse relationship between the number of EPC and the HbA1c in the patients ($R = -0.68$, $P = 0.01$, Fig. 2B).

No evidence for increased apoptosis in cultured EPC from type 1 diabetes patients

To evaluate if the reduction in the number of EPC was due to apoptosis, we analyzed the binding of Annexin V to phosphatidylserine in both EPC cultured for four 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 obtained by flow cytometry revealed no significant difference in the mean levels of Annexin V binding to EPC cultured from patients or controls 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 total MNC fraction in the patient group compared to that of the controls (370.9 ± 78.5 v.s. 304.7 ± 139.5 , $p = 0.17$).

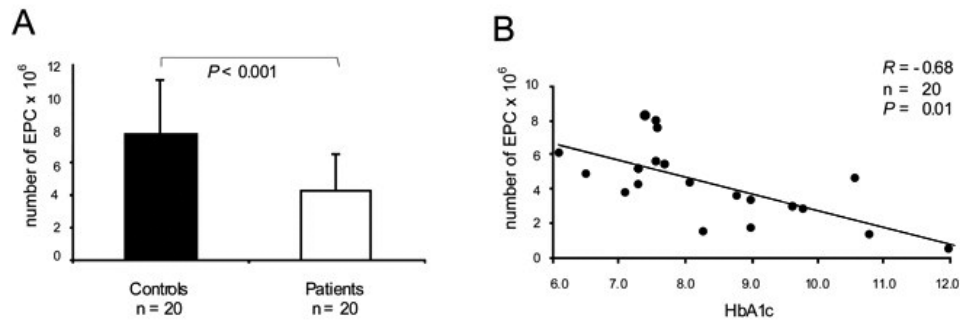


Figure 2.

MNC were isolated out of 60 ml peripheral blood from 20 controls and 20 type 1 diabetes patients. MNC were plated on gelatin-coated dishes and EPC were cultured for 4 days. The total EPC numbers were counted and a 44% reduction was seen in the type 1 diabetes patients compared to the healthy controls (A). This reduction in EPC numbers inversely correlated with glycemic control assessed by HbA1c (B).

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 factors⁵. 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 endothelial cells 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. HUVEC were seeded on extracellular matrix and subjected overnight to conditioned medium of diabetic EPC (n=10) or of EPC from age-matched controls (n=10). Conditioned media of healthy controls markedly stimulated tube formation when compared to non-conditioned media (EGM -/-) demonstrating that EPC can facilitate angiogenesis in a paracrine fashion (Fig. 3). In contrast, the conditioned media of the patient EPC significantly reduced tube formation when compared to non-conditioned media suggesting that cultured EPC from type 1 diabetes patients can secrete factors that impair angiogenesis *in vitro*.

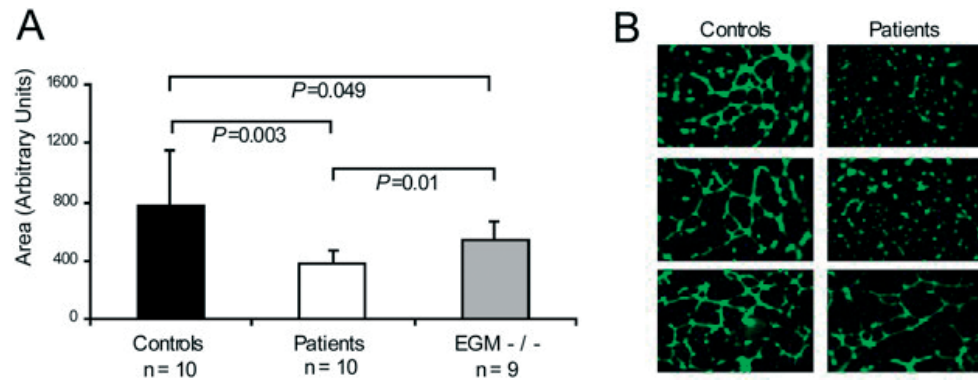


Figure 3.

Angiogenic capacity of conditioned media of EPC in an in vitro angiogenesis assay. HUVEC were plated on extra cellular 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 significant inhibition of tube formation of patient EPC conditioned media is shown (A). Representative pictures of the stained tubule networks formed after incubation of HUVEC with the conditioned media of EPC derived from thee different patients and their age-gender matched controls (B).

Discussion

Our data support the hypothesis that EPC are dysfunctional in patients with type 1 diabetes. First, a reduced number of EPC could be cultured from the MNC fraction of type 1 diabetes patients when compared to healthy control subjects. A reduction of the number of EPC in patients with risk factors for coronary artery disease has also been reported by others⁶⁻⁸. Our data are consistent with a previous report by Schatteman *et al.*⁷ who showed that CD34⁺ cells derived from type 1 diabetes patients produced less differentiated endothelial cells than their non-diabetic derived counterparts. Here, we have extended their observation by showing that in type 1 diabetes, EPC numbers inversely correlate with HbA1c levels demonstrating 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 apoptosis¹¹ and increased apoptosis has been associated with the adverse metabolic state and oxidative stress in diabetes¹², we hypothesized that increased apoptosis

could explain the reduced EPC numbers. When we analyzed early apoptosis in EPC cultured for four 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 diabetes patient group compared to the control group. These data suggest that increased apoptosis is unlikely to be involved in the reduction of the number of EPC in type 1 diabetes 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 EPC is likely to impact on vascular integrity as it was recently reported that, in healthy men, the number of EPC serve as a surrogate marker for vascular function and cumulative cardiovascular risk⁹.

Having established that the number of EPC is reduced in type 1 diabetes, we determined if the function of the remaining cells was altered compared when compared to the controls. So far, only few studies addressed the subject of EPC dysfunction. It was reported that EPC isolated from patients with CAD displayed an impaired migratory response⁶ and that, in type 2 diabetes, EPC adhesion to stimulated endothelial cells is impaired⁸. Although EPC enhance new vessel formation, these cells do not form the entire vessel *de novo* and the process always includes mature endothelial cells¹³. Recently, it has been suggested that a major function of EPC could be the secretion of angiogenic factors to activate resident mature EC¹⁴. Here we show that the angiogenic capacity of conditioned media from patient-derived EPC is not only reduced, but it may even contain an inhibitor for *in vitro* tube formation of endothelial cells.

Furthermore, our data demonstrate that type I diabetes is associated with altered EPC function and that these changes are observed even though the EPC 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 EPC we have performed preliminary Affymetrix DNA microarray analyses. Indeed, we observed extensive differential gene expression in patient-derived cultured EPC compared to control subjects. Interestingly, many of these alterations have been reported to be associated with diabetes mellitus in general, hyperglycemia or oxidative stress such as plasminogen activator inhibitor 1¹⁵ and osteopontin¹⁶. This demonstrates that may EPC function as “bio-sensors”, translating metabolic cues into altered gene expression. How the EPC “remembers” its metabolic descent in culture is unknown and needs further investigation.

Taken together, we demonstrate that EPC cultured from type 1 diabetes 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}. EPC or MNC 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.

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