Human Endothelial Progenitor Cells From Type II Diabetics Exhibit Impaired Proliferation, Adhesion, and Incorporation Into Vascular Structures

Oren M. Tepper, BA; Robert D. Galiano, MD; Jennifer M. Capla, BA; Christoph Kalka, MD; Paul J. Gagne, MD; Glen R. Jacobowitz, MD; Jamie P. Levine, MD; Geoffrey C. Gurtner, MD

Background—The recent discovery of circulating endothelial progenitor cells (EPCs) has altered our understanding of new blood vessel growth such as occurs during collateral formation. Because diabetic complications occur in conditions in which EPC contributions have been demonstrated, EPC dysfunction may be important in their pathophysiology.

Methods and Results—EPCs were isolated from human type II diabetics (n=20) and age-matched control subjects (n=20). Proliferation of diabetic EPCs relative to control subjects was decreased by 48% (P<0.01) and inversely correlated with patient levels of hemoglobin A1C (P<0.05). Diabetic EPCs had normal adhesion to fibronectin, collagen, and quiescent endothelial cells but a decreased adherence to human umbilical vein endothelial cells activated by tumor necrosis factor-α (TNF-α) (P<0.05). In a Matrigel assay, diabetic EPCs were 2.5 times less likely to participate in tubule formation compared with controls (P<0.05).

Conclusions—These findings suggest that type II diabetes may alter EPC biology in processes critical for new blood vessel growth and may identify a population at high risk for morbidity and mortality after vascular occlusive events.

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Key Words: collateral circulation • diabetes mellitus • angiogenesis

Diabetes mellitus is associated with both an increased risk of atherosclerotic disease and poor outcomes after vascular occlusion. The clinical severity of vascular occlusive disease in diabetics has in part been attributed to impaired collateral vessel development.¹ Current explanations for impaired blood vessel growth (neovascularization) in diabetics have involved the conventional paradigm of angiogenesis and focused on alterations in mature endothelial cells and monocytes.²,³

There is, however, increasing evidence that neovascularization in adults is not solely the result of angiogenesis but may also involve bone marrow–derived endothelial progenitor cells (EPCs) in the process of vasculogenesis.⁴ Unlike angiogenesis, little is known about the effect of diabetes on vasculogenesis and its major effector cell, the EPC. It has been shown that patients at risk for coronary artery disease have decreased numbers of circulating EPCs with impaired activity,⁵ and recent studies also suggest that vasculogenesis may be impaired in animal models of diabetes mellitus.⁶

The effects of diabetes on EPCs may be clinically important because common vascular complications associated with diabetes are conditions in which EPC contributions are now well established.⁷ It is known that EPCs contribute up to 25% of endothelial cells in newly formed vessels in animal models.⁸ The present study examined diabetic EPCs in processes critical for blood vessel growth to elucidate specific mechanisms that may account for the poor outcomes observed in diabetics after vascular ischemic and occlusive events.

Methods

Patient Characteristics

Peripheral blood samples were collected from type II diabetics (n=20) or nondiabetic control subjects (n=20) in full accordance with the New York University Medical Center Institutional Review Board. Clinical data for diabetic and control patients are shown in the Table. Volunteers were matched in terms of comorbidity and medications, and all were free of wounds, ulcers, or recent surgery that may influence EPC kinetics.⁹

EPC Isolation

EPCs were cultured according to previously described techniques.¹⁰,¹¹ Briefly, mononuclear cells (MNCs) were isolated by density centrifugation (Histopaque 1077). After purification with 3 washing steps, 10¹⁰ or 2×10⁹ MNCs were plated on fibronectin-coated, 6-well plates or 4-well glass slides, respectively. Cells were
cultured in endothelial cell basal medium-2 (Clonetics) supplemented with EGM-2 MV single aliquots consisting of 5% FBS, vascular endothelial growth factors (VEGF), fibroblast growth factor-2, epidermal growth factor, insulin-like growth factor-1, and ascorbic acid. The 6-well plates were replated at day 4, and all assays were performed by using cells harvested on day 7 with PBS plus 5 mmol/L EDTA.

EPC Characterization

Endothelial identity was confirmed by fluorescent-activated cell sorting (FACS) with antibodies recognizing human vascular endothelium (VE)-cadherin (Chemicon), FITC-conjugated CD34 (Pharmingen), PE-conjugated CD31 (Pharmingen), FITC-conjugated KDR (Sigma), and PE-conjugated P1H12 (Chemicon). All antibodies were added to 2×10⁵ cells for 30 minutes at 4°C, and a FITC-conjugated anti-mouse antibody (Vector) was added for staining with VE-cadherin. After incubation, cells were fixed with 1% paraformaldehyde, and quantitative analysis was performed on a FACStar flow cytometer (Becton Dickinson) measuring 20,000 cells per sample. For fluorescent labeling of EPCs, cells were incubated with diododecyl-tetramethylindolo-carbocyanine perchlorate (diI) (Molecular Probes) at a concentration of 2.5 μg/mL PBS for 5 minutes at 37°C and 15 minutes at 4°C.

Ischemia Model

A standardized, reproducible in vivo model of soft tissue ischemia was achieved by creating a U-shaped peninsular incision (1.25 cm in width and 2.5 cm in length) on the back of athymic nude mice (Jackson) penetrating the skin, dermis, and underlying adipose tissue. This tissue was elevated from the underlying muscular bed, and the degree of ischemia within the tissue was determined with EPCs from our study population (ie, older individuals with diabetes and vascular disease) are capable of incorporating into blood vessels, we injected a sample of EPCs from our control group into mice with an induced gradient of ischemia (Figure 1, C

Patient Characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Type II Diabetics</th>
<th>Control Subjects</th>
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<tbody>
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Values are percentages or mean±SD.

EPC Adhesion to Matrix Molecules

A collagen mixture (Vitrogen) (100 μg/mL) or fibronectin (100 μg/mL) was coated onto 24-well plates for 2 hours at 37°C. Wells were blocked with 1% BSA in PBS for 2 hours and EPCs (1×10⁵) were added to each well to attach for 1 hour. Adherent cells were stained with 0.1% crystal violet and rinsed with 10% acetic acid to elute the stain from the cells. Attached cells were quantified by analyzing the optical density of the media at a wavelength of 600 nm with a microtiter plate reader.

EPC Adhesion to Endothelial Cells

A monolayer of human umbilical vein endothelial cells (HUVECs) was prepared 48 hours before the assay by plating 2×10⁵ cells (passage 5 to 8) in each well of 4-well glass slides. HUVECs were pretreated for 12 hours with TNF-α (BD Biosciences) (1 ng/mL) or media. EPCs were labeled with diI and 1×10⁶ cells were added to each well and incubated for 3 hours at 37°C. Nonattached cells were gently removed with PBS, and adherent EPCs were fixed with 4% paraformaldehyde and counted in 10 random fields.

Matrigel Tubule Assay

Matrigel (Sigma) was thawed and placed in 4-well glass slides at room temperature for 30 minutes to allow solidification. Dil-labeled EPCs (2×10⁴) were coplated with 4×10⁴ human microvascular endothelial cells (MVECs) and incubated at 37°C. Tubule formation was defined as a structure exhibiting a length 4 times its width. The proportion of EPCs in tubules was determined in 10 random fields.

Statistical Analysis

Statistical analysis was performed with a Student’s t test, and results are expressed as mean±SEM. Comparison of EPC function with diabetic duration and glycosylated hemoglobin (Hb A₁c) was performed by linear regression analysis. Probability values of P<0.05 were considered statistically significant.

Results

EPC Characterization

FACS analysis confirmed the endothelial phenotype of the EPCs used in the present study. The expression profile of cultured EPCs included KDR (71.8±7.4%), VE-cadherin (75.5±5.3%), P1H12 (79.3±8.2%), CD34 (22.4±10.1), and CD31 (79.1±5.2%). In addition, cultured EPCs were found to endocytose acLDL and bind UEA-1 lectin (Figure 1, A and B). Previous studies characterizing EPCs have disclosed that they do not express markers of T lymphocytes or macrophages.

Ability of EPCs to Incorporate Into Vessels Within Ischemic Tissue

It has been shown that bone marrow–derived EPCs contribute up to 27% of endothelial cells in an in vivo Matrigel assay and 18% of endothelial cells in a corneal neovascularization model. Systemic delivery of circulating EPCs from young, healthy volunteers to nude mice can further augment neovascularization, and transplanted cells can be found in up to 50% of capillaries in ischemic zones. To confirm that EPCs derived from our study population (ie, older individuals with vascular disease) are capable of incorporating into blood vessels, we injected a sample of EPCs from our control group into mice with an induced gradient of ischemia (Figure 1, C

Type II Diabetics

Control Subjects

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through E)). This demonstrated that injected human EPCs were incorporated into new blood vessels located within the ischemic zone (Figure 1, F and G).

EPC Proliferation
Diabetic cultures contained significantly fewer EPCs at day 7 compared with nondiabetic control subjects (41.3±4.1 versus 79.5±7.4 EPCs/×200 field; \( P<0.01 \)), and this inversely correlated with Hb A1c levels in diabetic patients (\( R=-0.344, P<0.05 \)) (Figure 2, A through D). Significantly fewer EPC-bearing clusters were also found in diabetic cultures compared with controls (8.6±4.4 versus 17.7±3.6, respectively; \( P<0.05 \)). The number of clusters inversely correlated with the number of years diagnosed with diabetes (\( R=-0.471, P<0.01 \)) (Figure 2, E and F).

EPC Adhesion to Matrix Molecules and Mature Endothelial Cells
We assessed the adhesion of normal and diabetic EPCs, because adhesion to both activated endothelial cells and the extracellular matrix is believed to be important during new blood vessel growth. Diabetic EPCs were found to be significantly impaired in their ability to adhere to a HUVEC monolayer activated with TNF-\( \alpha \) (33.5±3.9 versus 55.2±3.6 EPCs/0.25 mm\(^2\); \( P<0.05 \)) but exhibited normal adhesion to quiescent HUVECs (23.8±2.1 versus 27.6±1.9 EPCs/0.25 mm\(^2\), respectively; \( P=0.37 \)). No significant differences were found in the adhesion of diabetic and control EPCs to fibronectin (0.464±0.13 versus 0.503±0.15 OD; \( P=0.81 \)) and collagen (0.535±0.08 versus 0.612±0.02 OD; \( P=0.33 \)) (Figure 3).

Matrigel Tubule Assay
A Matrigel tubule assay was performed to investigate the ability of EPCs to integrate into vascular structures. Cocultures of EPCs and MVECs within Matrigel led to the formation of an extensive tubule network in both diabetic and control samples. Fluorescent tagging of EPCs with DiI enabled delineation from MVECs, and analysis under fluorescence revealed that fewer diabetic EPCs were incorporated into tubules when compared with control EPCs (14.8±6% versus 37±7%, respectively; \( P<0.05 \)) (Figure 4).

Discussion
There is strong evidence that EPCs play a significant role in neovascularization, particularly during ischemic conditions.
This study examined EPC function in diabetes, a disease in which impairments in ischemia-induced neovascularization have been described. We demonstrate that diabetic EPCs exhibit alterations in functions important for blood vessel growth and may represent a mechanism by which diabetics are impaired in their ability to form collaterals.

The adhesion assays provide insight into the mechanism by which EPCs function in vivo, through initial attachment to

**Figure 2.** EPC proliferation. Representative pictures of dual-stained EPCs (acLDL and lectin) from control cultures (A) and diabetic cultures (B) after 1 week. Quantification of these cultures revealed that diabetic EPCs have significant impairment in proliferation (C) that inversely correlated with Hb A1c levels (D). In addition, diabetic cultures contained significantly fewer EPC-bearing clusters (E), which inversely correlated with the total number of years with diabetes (F).

**Figure 3.** EPC adhesion. EPCs were labeled with a Dil fluorescent marker (red) and allowed to adhere to a monolayer of HUVECs (blue; nuclear stain 4',6-diamidino-2-phenylindole/DAPI) for 3 hours. Representative pictures illustrating adhesion of control EPCs (A) and diabetic EPCs (B) to TNF-α-activated HUVECS are shown. Significantly fewer diabetic EPCs adhered to activated HUVEC monolayers (C). No differences in adhesion to either fibronectin or collagen were noted between diabetic EPCs and controls (D).
endothelium-lined vessels (cell-cell adhesion) and then extension into the interstitium (cell-matrix adhesion). No significant difference was noted in the adhesion of diabetic and control EPCs to specific matrix molecules. However, the finding that diabetic EPCs bind poorly to TNF-α–activated endothelial cells, as would occur in the initial phases of inflammation, suggests that diabetic EPCs are initially recruited less avidly in vivo.

Current data suggest that EPCs work in concert with existing endothelial cells to form vessels rather than forming entirely new vessels de novo. In this study, we demonstrated gross impairments in the ability of diabetic EPCs to incorporate into tubules with MVECs. It is interesting that the magnitude of impairment of diabetic EPCs was greatest in the Matrigel assay. Because this is a global assay evaluating multiple cellular processes involved in blood vessel growth, it is likely that other unexamined components of blood vessel growth may be impaired in diabetes.

Under normal conditions, the number of circulating EPCs is relatively small but is increased in response to trauma or ischemia, which mobilizes these cells from the bone marrow and allows them to proliferate. Using culture conditions that favor EPC growth, we show that diabetic EPCs proliferate less than those from nondiabetic patients. This difference inversely correlated with Hb A1c levels and may provide insight into the relation between tight glucose control and EPC function. Others have used an analogous technique to examine circulating EPC levels in patients with coronary artery disease. In their diabetic population, similar trends were found but did not reach statistical significance. The greater number of diabetics enrolled in our study as well as the longer culture period (7 versus 4 days) may have produced this difference.

There is evidence to suggest that statins (HMG-CoA–reductase inhibitors) stimulate EPC kinetics. In particular, Dammel and colleagues have reported in patients with coronary artery disease that 4 weeks of statin therapy resulted in increased circulating EPCs. Because 55% of diabetics and 25% of control subjects in the present study were receiving statin medication at the time of blood donation, one would expect our data to underestimate differences in proliferation between the two groups. However, when patients receiving statins were compared with those not receiving statin medication within each group, no differences in EPC proliferation were noted (data not shown). This finding may have resulted from differences in the duration of therapy in our study population (months-to-years) versus other studies (weeks). Because most studies that have looked at the relation between statins and EPCs have also been restricted to short-term durations of treatment, this raises the question of whether the beneficial effects of statin therapy on EPC biology may be transient.

Many of the diabetic patients who have impaired blood vessel growth after ischemic events also have increased retinal neovascularization (diabetic retinopathy). This so-called “diabetic paradox” is at present poorly understood. The question of how EPC dysfunction may participate in diabetic retinopathy is especially intriguing, given the recent findings by Grant et al that bone marrow–derived EPCs play a role in a new model of adult retinal revascularization. A plausible explanation may be that the retina responds differently to ischemic events when compared with tissue outside of the central nervous system. VEGF is known to be significantly elevated in the ocular fluid of diabetic patients but has also been shown to be decreased in ischemic nonretinal tissues. Because VEGF has a stimulatory effect on EPC proliferation and is a potent stimulator of vasculogenesis, unique retinal pathways for VEGF regulation may be sufficient to overcome EPC dysfunction resulting in diabetic retinopathy. Work currently underway in our laboratory supports this notion and demonstrates that nonretinal VEGF administration reverses impaired wound healing in diabetic animal models by mobilizing and attracting bone marrow–derived EPCs (unpublished data).

It is important to note that no single definition exists for EPCs, and many researchers are currently working to clearly elucidate the exact lineage and differentiation of vascular
stem cells. Studies using EPCs isolated by identical techniques demonstrate that these cells incorporate into vascular structures in vivo, whereas fully differentiated endothelial cells do not.4,18 The observation that EPC administration increases neovascularization has generated interest in using autologous cells to restore blood flow in patients with vascular disease. Our studies suggest that if such techniques are to reach clinical fruition, one must also account for the functionality of the transplanted cells. Current studies correlating long-term outcome with EPC numbers and function may help identify subsets of diabetic patients at high risk after vascular occlusive events.

As the clinical relevance of adult vasculogenesis becomes clear, EPC function will become increasingly important during the in vivo response to ischemia. We illustrate here that EPCs from type II diabetics are impaired in adhesion to endothelium (critical for bringing circulating EPCs to a halt), proliferation (important for amplifying the pool of endothelial cells), and tubulization (necessary to create new vascular structures). Such abnormalities in EPC function may account for the impaired neovascularization observed in diabetic patients.

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