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.

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, 10^6 MNCs were plated on fibronectin-coated, 6-well plates or 4-well glass slides, respectively. Cells were...
EPC-bearing clusters, which may represent correlates of embryonic blood-islands,4 were also counted.

### 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.15 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 × 10⁵ 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 × 10⁵ 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.11

### 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.8 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.16 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
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.17 Diabetic EPCs were found to be significantly impaired in their ability to adhere to a HUVEC monolayer activated with TNF-α (33.5 ± 3.9 versus 55.2 ± 3.6 EPCs/0.25 mm²; *P* < 0.05) but exhibited normal adhesion to quiescent HUVECs (23.8 ± 2.1 versus 27.6 ± 1.9 EPCs/0.25 mm², 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.18
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).
growth may be impaired in diabetes. It is likely that other unexamined components of blood vessel multiple cellular processes involved in blood vessel growth, it is measured using a Matrigel assay. Because this is a global assay evaluating the magnitude of impairment of diabetic EPCs was greatest in the rate into tubules with MVECs. It is interesting that the gross impairments in the ability of diabetic EPCs to incorporate into existing endothelial cells to form vessels rather than forming entirely new vessels de novo. In this study, we demonstrated substantial contribution to tubule networks with MVECs (A). This is more clearly shown in a high-power view (B). In contrast, diabetic EPCs showed minimal incorporation into the developing vascular network shown in low-power (C) and high-power views (D). Quantification of EPC tubulization revealed that significantly fewer diabetic EPCs were incorporated into tubules (E).

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 is 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, Dimmeler 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 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.
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. 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.

Acknowledgments
This study was supported by the Juvenile Diabetes Research Foundation (O.M. Tepper), the Deutsche Forschungsgemeinschaft (SFB612) (Dr Kalka), and the Saroff Endowment for Cardiovascular Research (Dr Gurtner). The authors thank Marie Frisone for assistance in preparation of the manuscript and Dr Hooman Soltanian for statistical analysis.

References
Human Endothelial Progenitor Cells From Type II Diabetics Exhibit Impaired Proliferation, Adhesion, and Incorporation Into Vascular Structures
Oren M. Tepper, Robert D. Galiano, Jennifer M. Capla, Christoph Kalka, Paul J. Gagne, Glen R. Jacobowitz, Jamie P. Levine and Geoffrey C. Gurtner

Circulation. 2002;106:2781-2786; originally published online November 4, 2002;
doi: 10.1161/01.CIR.0000039526.42991.93
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/106/22/2781

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/