

# Nitric Oxide Cytoskeletal-Induced Alterations Reverse the Endothelial Progenitor Cell Migratory Defect Associated With Diabetes

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**Stromal-derived factor-1 (SDF-1) is a critical chemokine for endothelial progenitor cell (EPC) recruitment to areas of ischemia, allowing these cells to participate in compensatory angiogenesis. The SDF-1 receptor, CXCR4, is expressed in developing blood vessels as well as on CD34+ EPCs. We describe that picomolar and nanomolar concentrations of SDF-1 differentially influence neovascularization, inducing CD34+ cell migration and EPC tube formation. CD34+ cells isolated from diabetic patients demonstrate a marked defect in migration to SDF-1. This defect is associated, in some but not all patients, with a cell surface activity of CD26/dipeptidyl peptidase IV, an enzyme that inactivates SDF-1. Diabetic CD34+ cells also do not migrate in response to vascular endothelial growth factor and are structurally rigid. However, incubating CD34+ cells with a nitric oxide (NO) donor corrects this migration defect and corrects the cell deformability. In addition, exogenous NO alters vasodilator-stimulated phosphoprotein and mammalian-enabled distribution in EPCs. These data support a common downstream cytoskeletal alteration in diabetic CD34+ cells that is independent of growth factor receptor activation and is correctable with exogenous NO. This inability of diabetic EPCs to respond to SDF-1 may contribute to aberrant tissue vascularization and endothelial repair in diabetic patients. *Diabetes* 55: 102–109, 2006**

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Received for publication 23 June 2005 and accepted in revised form 5 October 2005.

CKD, chronic kidney disease; DETA/NO, diethylenetriamine NONOate; EPC, endothelial progenitor cell; FITC, fluorescein isothiocyanate; HREC, human retinal endothelial cell; SDF-1, stromal-derived factor-1; VASP, vasodilator-stimulated phosphoprotein; VEGF, vascular endothelial growth factor.

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**S**tromal derived factor-1 (SDF-1) is a small cytokine belonging to the C-X-C subfamily that was originally isolated from a murine bone marrow stromal cell line (1). SDF-1 and its receptor, CXCR4, are essential for normal ontogeny of hematopoiesis during embryogenesis (2) and play a critical role in lymphopoiesis and myelopoiesis in the adult (3,4). SDF-1 has been shown to be upregulated in the myocardium under ischemia (5) and plays a critical role in homing and chemotaxis of CXCR4-expressing progenitor cells. The ability of SDF-1 to induce chemotaxis has been shown to be regulated by the activity of CD26, a cell-surface dipeptidyl peptidase that degrades SDF-1 (6). Endothelial progenitor cells (EPCs) derived from the bone marrow are modulated by SDF-1.

The stem cell marker CD34 identifies one potential source of EPCs (7). CD34+ mononuclear cells, after 7 days of culture on fibronectin, display an endothelial cell phenotype, are able to incorporate acetylated LDL, produce nitric oxide (NO) when stimulated with vascular endothelial growth factor (VEGF), and express platelet/endothelial cell adhesion molecule-1 and Tie-2 receptor (8).

We have previously demonstrated that SDF-1 levels are increased in the vitreous of diabetic patients with retinopathy and macular edema and that the levels correlate with the severity of diabetic retinopathy (9). In experimental animal models, we have also shown that EPCs play a central role in the development of proliferative retinopathy (10). In these studies, we investigated the ability of diabetic and control CD34+ cells to respond to SDF-1 and the mechanism of the defective response of diabetic CD34+ cells.

## RESEARCH DESIGN AND METHODS

**Tissue culture and reagents.** All tissue culture reagents were obtained from Invitrogen (Carlsbad, CA) and MediaTech (Herndon, VA). SDF-1 was obtained from R&D Systems (Minneapolis, MN). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated.

**Isolation of CD34+ cells.** The study protocol was approved by the institutional review board at the University of Florida, and written informed consent was obtained from each patient. Blood was collected from 18 patients with stage V chronic kidney disease (CKD) (patients requiring hemodialysis) as a result of type 2 diabetes and 21 diabetic patients with stage I or II CKD (patients with an estimated glomerular filtration rate  $\geq 60$  ml/min). Ten of these patients had type 1 and 11 had type 2 diabetes and 19 were normal control subjects. Blood was collected by routine venipuncture into CPT tubes

with heparin (BD Biosciences, Franklin Lakes, NJ). For hemodialysis patients, the blood was collected before hemodialysis. After centrifugation at room temperature in a swinging bucket rotor for 20 min at 1,800g, the mononuclear cells were diluted with PBS supplemented with 2 mmol/l EDTA. The cells were centrifuged for 10 min at 300 RCF and the cell pellet washed; this procedure was repeated once. Each  $3.3 \times 10^7$  cells peripheral blood mononuclear cells was resuspended in 100  $\mu$ l PBS supplemented with 2 mmol/l EDTA, to which 33  $\mu$ l of FcR-blocking reagent (Miltenyi Biotec, Auburn, CA) and 33  $\mu$ l of magnetic microbeads conjugated with an anti-CD34 antibody were added. After incubation for 30 min at 4°C, the cells were diluted in 10  $\times$  volume of PBS supplemented with 2 mmol/l EDTA supplemented with 0.1% BSA. The CD34+ cells were positively selected using an automated magnetic selection autoMACS (Miltenyi Biotec). The selected cells were confirmed to be CD34+ cells by costaining with phycoerythrin-conjugated anti-CD34 (Miltenyi Biotec) and fluorescein isothiocyanate (FITC)-conjugated anti-CD45.

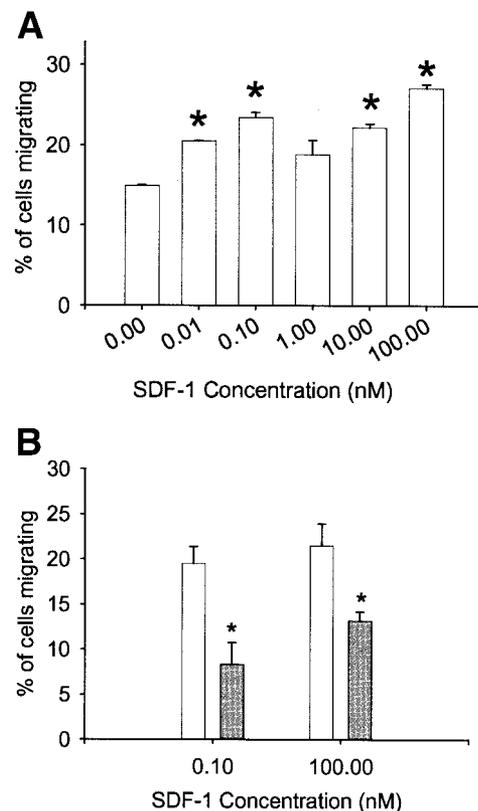
**Cell culture.** Primary cultures of human retinal endothelial cells (HRECs) were prepared and maintained as previously described (11,12), and cells in passages 2–5 were used in these studies. EPCs were cultured from peripheral blood as follows. Peripheral blood mononuclear cells were isolated as above and cultured on fibronectin-coated plates for 6 days in EndoCult Medium (StemCell Technologies) before being treated with 0 or 100 pmol/l or 100 nmol/l of SDF-1 for 24 h. The endothelial nature of the cells was confirmed, after culturing for 6 days, with incorporation of fluorescently labeled acetylated LDL and tetrahydrodamine isothiocyanate-conjugated Ulex europaeus agglutinin-1.

**SDF-1-induced chemotaxis.** CD34+ cell chemotaxis was done by staining the cells with Calcein-AM (Molecular Probes) before loading them onto the Boyden Chamber. SDF-1 was loaded in the bottom chamber, which was overlaid with a polycarbonate membrane (8- $\mu$ m pores; Neuro Probe, Gaithersburg, MD) coated with 10% bovine collagen, and the cells were loaded in the top chamber. After 4.5 h at 5% CO<sub>2</sub> at 37°C, the percentage of cells that migrated was determined by collecting the media in the lower chamber and determining the relative fluorescence using a Synergy HT (Bio-Tek Instruments, Winooski, VT) with an excitation of  $485 \pm 20$  and an emission of  $528 \pm 20$ . Twenty-four-hour pretreatments with 100  $\mu$ mol/l diethylenetriamine NONOate (DETA/NO; Cayman Chemicals, Ann Arbor, MI), an NO donor, or 200  $\mu$ mol/l 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide potassium salt (Sigma), an NO scavenger, were carried out in the CPT tubes, after centrifugation. Diprotein A, an inhibitor of dipeptidyl peptidase IV, (Alexis, San Diego, CA) was used where indicated at a concentration of 1 mmol/l and was added to the cells just before the migration assay. The 12G5 (R&D), a CXCR4-blocking antibody, was used at a concentration of 1  $\mu$ g/ml.

**Endothelial cell tube formation assay.** Endothelial tube formation was assessed on a synthetic basement membrane as per manufacturer's protocol (Matrigel; BD Biosciences). Briefly, the matrix was thawed overnight at 4°C and polymerized at 37°C for 30 min before use. HRECs were resuspended either in full endothelial cell growth media (for positive control), reduced serum media, or reduced serum media with 100 pmol/l or 100 nmol/l SDF. The cells were then seeded ( $3 \times 10^3$ ) on the Matrigel and the plates placed in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. Identical fields in each well were photographed every 12 h after plating. The photographs were digitized, and image-analysis software (Image; Scion, Frederick, MD) was used to measure total tube length and branch points. All conditions were tested in duplicate wells in three separate experiments using cells from different donors. EPC tube formation was performed after 6 days of culture.

**CD26/dipeptidyl peptidase IV activity.** The activity of CD34+-sorted peripheral blood mononuclear cells was measured in 384-well microplates using the chromogenic substrate Gly-Pro-p-nitroanilide (Gly-Pro-pNA; Sigma-Aldrich) (13). After labeling the CD34+ cells with Calcein-AM as above, a fixed number of isolated CD34+ cells were incubated at 37°C in the presence of 2 mmol/l Gly-Pro-pNA in 100  $\mu$ l PBS buffer (pH 7.4) containing 10 mg/ml of BSA. The amount of nitroanilide (pNA) formed in the supernatant was determined by readings performed every 5 min at 405 nm using a Synergy HT. The results were plotted as nanomoles of pNA released per  $1 \times 10^6$  cells released per minute, and the slope was calculated at the linear portion of the curve. Samples were run in triplicate.

**Micropipette technique setup.** The overall setup to analyze cell deformability was as previously described (14), consisting of a micropipette, a chamber on an inverted interference contrast microscope, two water reservoirs connected for micropipette hydrostatic pressure control, and a video system. Micropipettes were constructed from glass capillary tubes with an outer diameter of 1 mm and an inner diameter of 0.5 mm (A-M Systems, Everett, Washington). The micropipettes used had an average inner diameter of 5.4  $\mu$ m. Resting lymphocytes had diameters ranging from 5 to 17  $\mu$ m. Before use, micropipettes were flushed with 50  $\mu$ l of plasma to prevent adhesion to the glass surface. Two typical types of experiment, aspiration and recovery



**FIG. 1.** CD34+ cells have a biphasic response to increasing concentrations of SDF-1. **A:** Percentage of isolated CD34+ cells migrating to increasing concentrations of SDF-1. \* $P < 0.01$  compared with 0 nmol/l SDF-1. Each point done in duplicate shown  $\pm$  SE. Shown is an example of the migration pattern seen in 18 healthy volunteers. **B:** Percentage of CD34+ cells migrating to 100 pmol/l or 100 nmol/l of SDF-1 in the presence (■) or absence (□) of 12G5, a CXCR4 blocking antibody. \* $P < 0.01$  compared with migration in the absence of antibody. Each point done in triplicate shown  $\pm$  SE. Shown are representative results of three different experiments.

when possible, were performed to determine the mechanical properties of individual cells.

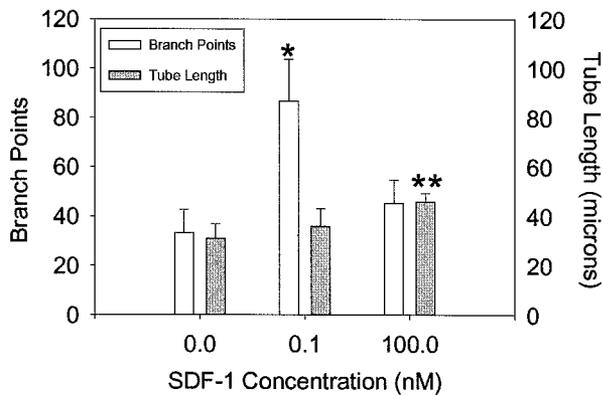
**Immunohistochemistry.** After fixing the EPCs with 3.7% paraformaldehyde and 0.2% Triton-X in PBS for 30 min at 37°C, the cells were washed with PBS and incubated in blocking solution (1% BSA in PBS) for 30 min at 37°C. The cells were then incubated with 10  $\mu$ g/ml of mouse antivasodilator-stimulated phosphoprotein (VASP) (BD Biosciences Pharmingen) or isotype controls in blocking solution. After an hour at room temperature, the cells were rinsed with PBS and incubated with secondary antibody FITC-conjugated anti-mouse (Southern Biotech) for 30 min; for VASP staining, the secondary was used at a concentration of 10  $\mu$ g/ml, for mammalian-enabled staining, the secondary was used at 5  $\mu$ g/ml. The cells were then rinsed and imaged.

**Fluorescence-activated cell sorter analysis of phosphorylated VASP.** CD34+ cells after isolation and DETA/NO treatment were fixed with methanol-free formaldehyde (91.5%) for 5 min before being diluted with PBS and permeabilized for 10 min with Triton X-100 (0.2% final). Portions of the samples were stained at room temperature for 45 min with a FITC-labeled antibody against phosphorylation of Ser<sup>239</sup> (16C2 antibody [500 mg/ml]; Nanotools). A second aliquot was labeled with the FITC-labeled antibody after preincubation with a specific blocking phosphopeptide. The fluorescence of these cells was used to determine the parameters for background fluorescence.

**Statistical analysis.** Statistical analysis was carried out using Student's *t* test and the Mann-Whitney rank-sum test.

## RESULTS

**CD34+ cells and microvascular endothelial cells respond to physiologic concentrations of SDF-1.** In previous work, we have demonstrated that SDF-1 is elevated in the vitreous fluid of diabetic patients with reti-



**FIG. 2.** SDF-1 promotes capillary tube formation at nanomolar and picomolar concentrations. HRECs were added to Matrigel-filled wells containing HRECs in serum-free media without SDF-1 or supplemented with 100 pmol/l or 100 nmol/l of SDF-1. Triplicates of each condition were performed and three fields in each well were photographed 48 h after plating. Tube length and branch points were determined for each field and shown is the average  $\pm$  SE. \* $P < 0.05$  compared with number of branch points at 0 nmol/l of SDF-1; \*\* $P < 0.05$  compared with tube length at 0 nmol/l of SDF-1.

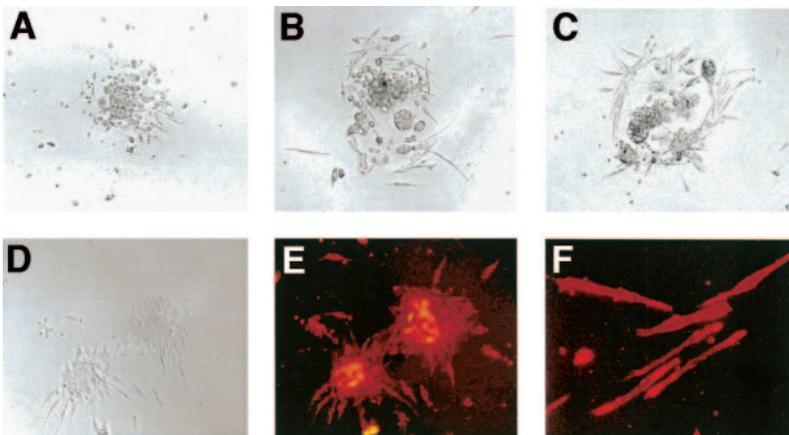
nopathy (9), and EPCs participate in pathological retinal angiogenesis (10). However, the concentration of SDF-1 in the vitreous of patients with proliferative diabetic retinopathy was  $179 \pm 172$  pmol/l (means  $\pm$  SD) (9). This is an SDF-1 concentration that is a full log lower than EPCs have previously been reported to migrate. It is well established that SDF-1 has profound effects at nanomolar concentrations, but the effects of picomolar concentrations have not been well documented. When picomolar concentrations were tested, CD34+ cells had a response to SDF-1 with two peaks of migration, one in the picomolar range and one in the nanomolar range (Fig. 1A). The response to SDF-1 at both the picomolar and nanomolar concentrations were mediated by the SDF-1 receptor, CXCR4, since the response was blocked to a similar extent by the CXCR4 blocking monoclonal antibody 12G5 (Fig. 1B). An SDF-1 bimodal effect on migration was also seen in HRECs as well as Jurkat cells (data not shown).

**Picomolar versus nanomolar concentrations of SDF-1 have unique effects on cell behavior.** To examine whether picomolar and nanomolar concentrations had similar efficacy on all properties of SDF-1, we tested its effects on tube formation. HRECs exposed to a 100-pmol/l concentration of SDF-1 demonstrated robust branching without a marked increase in tube length (Fig. 2). However, at 100 nmol/l SDF-1, tube length was statistically

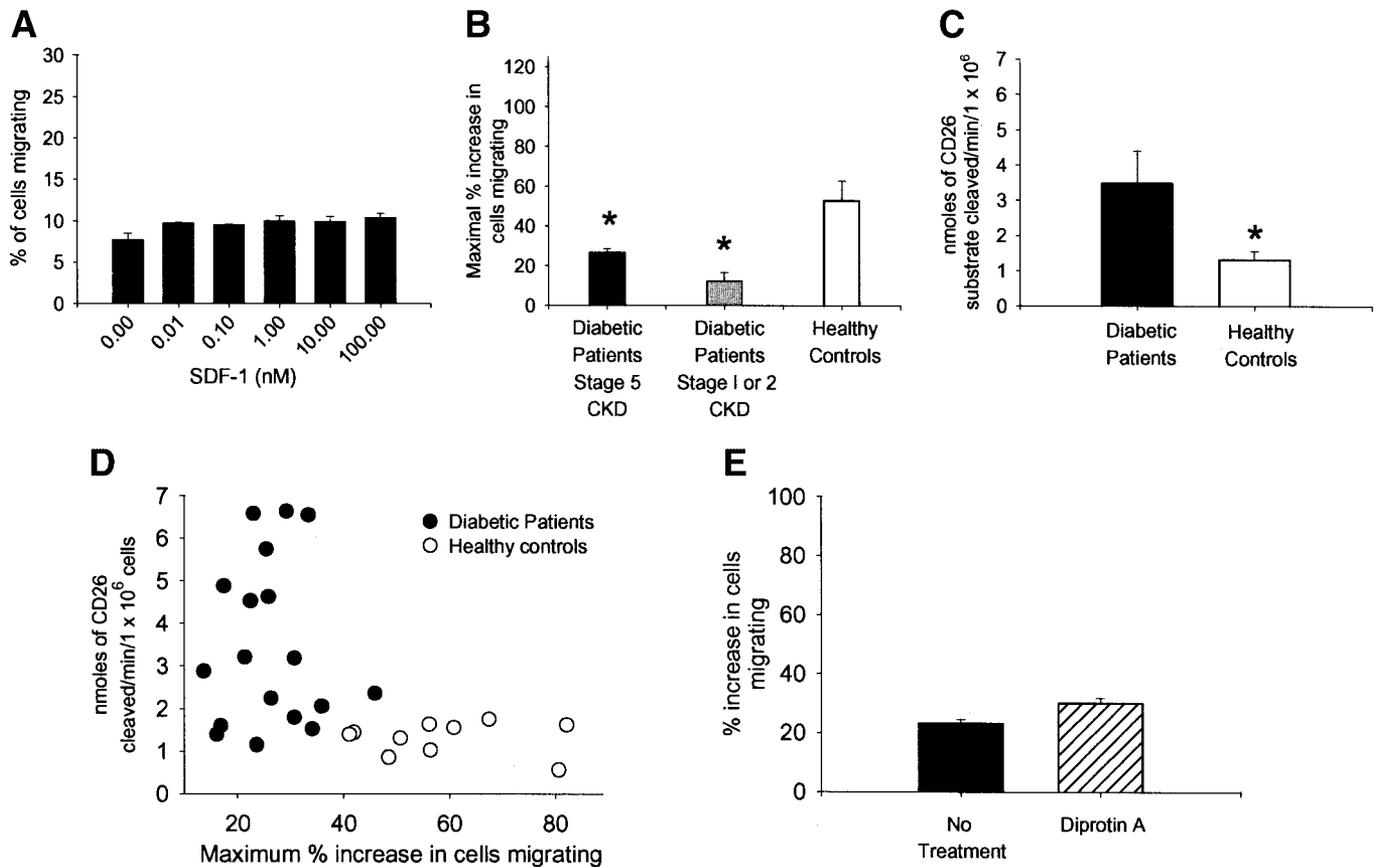
increased without increasing the number of branch points. SDF-1 promoted in vitro differentiation of CD34+ cells into EPCs and induced tube formation of cultured EPCs isolated from healthy control subjects at both concentrations of SDF-1 (Fig. 3).

**Diabetic patients have defective CD34+ cell migration in response to SDF-1.** If EPC migration to picomolar concentrations of SDF-1 found in the vitreous was the mechanism by which preretinal neovascularization occurred, one might predict that the EPCs isolated from diabetic patients would have enhanced migration at picomolar concentrations of SDF-1. Unexpectedly, CD34+ cells isolated from diabetic patients had markedly diminished migration, with a virtually flat response to SDF-1, over the picomolar and nanomolar concentrations tested (Fig. 4A). This was found in patients with retinopathy and stage V CKD or with stage I or II CKD (Fig. 4B). In addition, the result was the same whether the patients had type 1 or type 2 diabetes (data not shown). Compared with the maximal migration of CD34+ cells in normal control subjects, CD34+ cells from diabetic patients had ~30% of the maximal migratory activity (Fig. 4B).

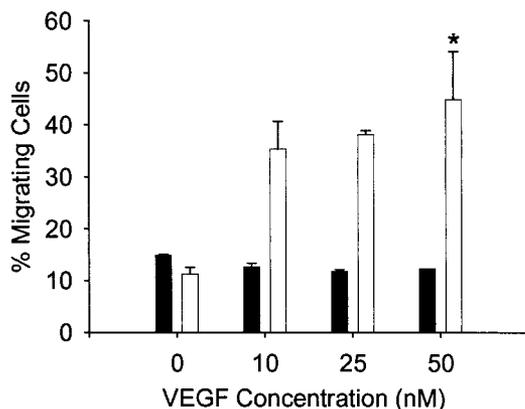
We reasoned that the defect in migration of CD34+ cells could be at the level of the receptor or due to downstream receptor effects. Previously, CD26/dipeptidyl peptidase IV was shown to cleave and inactivate SDF-1, blocking its ability to activate its receptor CXCR4 (13,15,16). To determine whether this was a possible mechanism for the decreased migration observed in CD34+ cells derived from diabetic individuals, we determined the level of CD26/dipeptidyl peptidase IV activity on these cells. We found twice the CD26/dipeptidyl peptidase IV activity present on CD34+ cells derived from diabetic patients versus control subjects (Fig. 4C). However, the increased level of dipeptidyl peptidase IV activity was not limiting migration. Even diabetic patients whose dipeptidyl peptidase IV activity was lower than that of normal control subjects (Fig. 4D) had defective migration. To confirm this, we used an inhibitor of dipeptidyl peptidase IV activity, diprotein A. Incubation of diabetic CD34+ cells with diprotein A, at concentrations that blocked 99% of the dipeptidyl peptidase IV activity (data not shown) increased migration, but the percent increase was still less than that seen in CD34+ cells isolated from normal control subjects (Fig. 4E). Consistent with the defect not being primarily at the receptor level, diabetic CD34+ cells did not migrate to VEGF, suggesting a more general inhibition of migration (Fig. 5).



**FIG. 3.** SDF-1 promotes EPC capillary tube formation at nanomolar and picomolar concentrations. EPCs after 1 week of culture were treated with 0 (A) or 100 (B) pmol/l or 100 nmol/l (C) of SDF-1 for 48 h. Shown are representative colonies. The cells' endothelial phenotype was confirmed with the colonies' (D) ability to incorporate tetrahydroamine isothiocyanate-acetylated LDL (E) and tetrahydroamine isothiocyanate-conjugated Ulex europaeus agglutinin-1 (F).



**FIG. 4.** CD34<sup>+</sup> cells isolated from diabetic patients on hemodialysis have a blunted response to increasing concentrations of SDF-1. **A:** Percentage of CD34<sup>+</sup> cells isolated from diabetic patients migrating to increasing concentrations of SDF-1. Shown is a representative graph of 1 of 27 patients studied. Each concentration was done in triplicate. Error bars  $\pm$  SE. **B:** The average maximal increase  $\pm$  SE in migration at any concentration of SDF-1 is shown for diabetic patients with stage V CKD, who are hemodialysis dependent (■;  $n = 18$ ), who are diabetic patients with stage I or II CKD, who have an estimated glomerular filtration rate  $>60$  ml/min (▣;  $n = 19$ ), and normal control subjects (□;  $n = 19$ ). Error bar  $\pm$  SE. \* $P$  value  $< 0.01$  compared with normal control subjects. **C:** The dipeptidyl peptidase activity of the isolated CD34<sup>+</sup> cells was determined in diabetic patients (■;  $n = 19$ ) and normal control subjects (□;  $n = 10$ ). The level of activity is expressed as nanomoles of dipeptidyl peptidase substrate cleaved per minute per  $1 \times 10^6$  cells. Error bars  $\pm$  SE. \* $P$  value  $< 0.01$  compared with diabetic patients on hemodialysis. **D:** CD26 activity does not explain the migratory defect in all diabetic patients. The dipeptidyl peptidase activity (expressed as nanomoles of dipeptidyl peptidase substrate cleaved per minute per  $1 \times 10^6$  cells) of all diabetic patients (■;  $n = 19$ ) and normal control subjects (○;  $n = 10$ ) is graphed according to their maximum migration response (maximum percent increase of cells migrating in response to any concentration of SDF-1). **E:** Inhibiting dipeptidyl peptidase activity with diprotein A does not normalize migration. Cells from diabetic patients with elevated levels of dipeptidyl peptidase activity were incubated with 1 mmol/l diprotein A (▨) or with vehicle (■) prior to and during migration to increasing concentration of SDF-1. Shown is a representative example of the maximal increase in migration to SDF-1 of one of three diabetic individuals tested. Samples run in duplicate. Bars indicate  $\pm$ SE.



**FIG. 5.** Diabetic CD34<sup>+</sup> cells do not respond to VEGF. Percentage of isolated CD34<sup>+</sup> cells migrating to increasing concentrations of VEGF. Each point done in duplicate shown  $\pm$ SE. Shown is an example of the migration pattern seen in a diabetic patient (■) and a healthy control subject (□). \* $P$  value  $< 0.05$  compared with migration at 0 nmol/l of VEGF.

**Cytoskeletal defect in diabetic CD34<sup>+</sup> cells.** Classically, the cytoskeletal structure has been investigated by cell deformability either by atomic force microscopy or micropipette technique (17,18). In the latter, micropipettes are made from 1-mm capillary-glass tubing pulled to a fine point by quick fracture to give a flat tip of desired diameter. Two typical types of experiment, aspiration and recovery, are usually performed to determine the mechanical properties of individual cells. Cell deformability can be assessed by aspirating a cell into a micropipette at a constant pressure and then measuring its deformation as a function of time. Using the micropipette technique, it has also been established that the cell rheological properties can be altered by disease and illness. Published data have shown alterations of the mechanical properties of erythrocytes in sickle cell disease (19), white blood cells in diabetes (14,20,21), and a variety of cells in cancer (22–25). CD34<sup>+</sup> cells isolated from patients with diabetes complications were extremely rigid; the deformability could not be determined because they could not be suctioned into the micropipette (Fig. 6). CD34<sup>+</sup> cells from normal con-

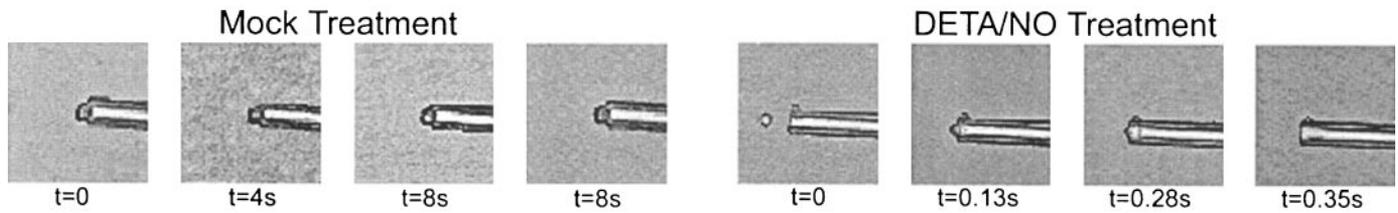


FIG. 6. CD34<sup>+</sup> cells isolated from diabetic patients were treated with a vehicle or DETA/NO for 24 h before testing their deformability by determining their ability to be suctioned into and expelled from a micropipette. After 14 s, the vehicle-treated CD34<sup>+</sup> cells can still be seen at the orifice of the micropipette, while the DETA/NO-treated cells start entering the micropipette at 0.067 s.

control subjects entered the micropipette very well (75% of cells completely entered the micropipette, 16.7% partially entered, and 8.3% could not be suctioned into the micropipette;  $n = 5$ ), while CD34<sup>+</sup> cells isolated from diabetic patients were not deformable (100% of the cells could not be suctioned into the micropipette;  $n = 6$ ). Previously, we have demonstrated a similar disturbance in white cells isolated from diabetic mice (14).

**CD34<sup>+</sup> cell migration is enhanced by incubation with an NO donor.** Local concentrations of NO dramatically affected hemangioblast behavior and changed vessel phenotype (26). Thus, we investigated whether the migratory defect of CD34<sup>+</sup> cells isolated from diabetic patients could be corrected by treatment with NO. Incubation of CD34<sup>+</sup> cells in the presence of an NO donor, DETA/NO, for 24 h increased the percentage of cells migrating in response to SDF-1 (Fig. 7). This enhancement was evident at picomolar and nanomolar concentrations of SDF-1. The effect of the NO donor on SDF-1 in stimulating CD34<sup>+</sup> cell migration was blocked by coincubating with the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (Fig. 7).

NO treatment also corrected the diabetic CD34<sup>+</sup> deformability defect (Fig. 6). While 100% of CD34<sup>+</sup> cells isolated from diabetic patients were not deformable when treated with vehicle, diabetic CD34<sup>+</sup> cells incubated with DETA/NO had a marked increase in deformability (85% of cells could fully enter the micropipette;  $n = 16$ ). This suggested that NO altered the cytoskeletal structure mediating the correction in the migration defect.

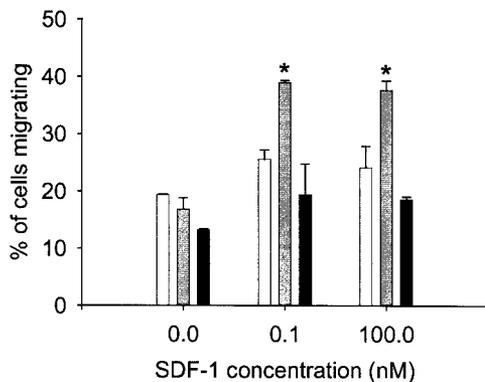


FIG. 7. Defect in migration of CD34<sup>+</sup> cells isolated from diabetic hemodialysis patients is corrected with an NO donor. Peripheral blood mononuclear cells from patients on hemodialysis secondary to diabetes were treated with 100  $\mu\text{mol/l}$  of an DETA/NO (▨), or 100  $\mu\text{mol/l}$  of an DETA/NO simultaneously with an NO scavenger (■), or mock treated (□) 24 h before isolation of CD34<sup>+</sup> cells and determining the percentage of cells that migrate to the indicated SDF-1 concentration. Each condition was done in triplicate and is representative of four different experiments. Shown is the average  $\pm$  SE. \* $P$  value < 0.05 compared with percentage of cells migrating with no treatment at the same SDF-1 concentration.

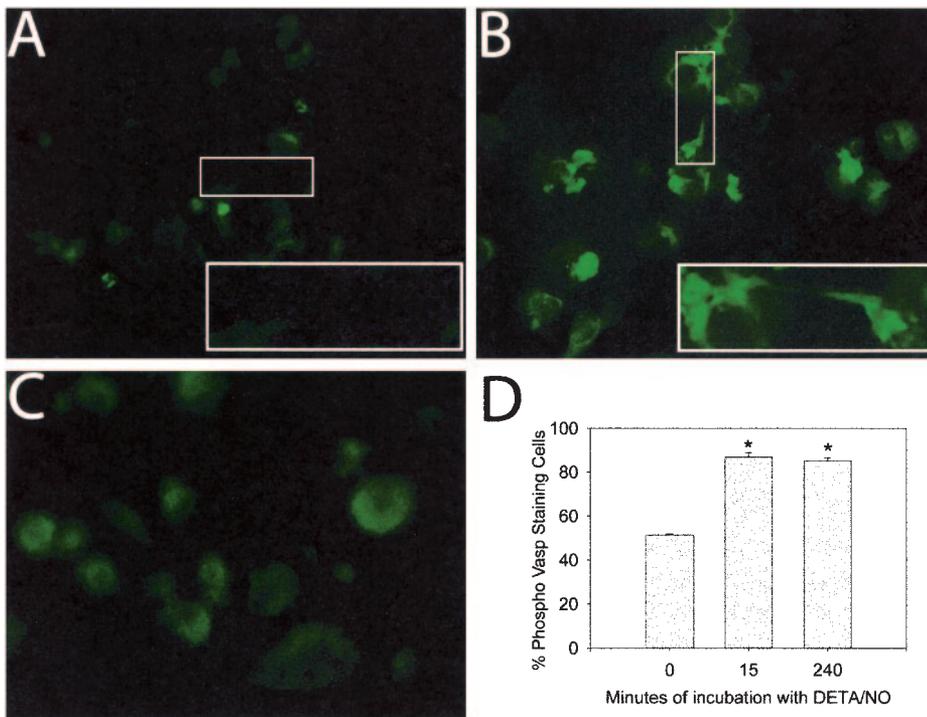
**NO causes redistribution of cytoskeletal motors facilitating migration.** To begin to elucidate the possible role that NO has on the cytoskeleton, we examined the distribution of VASP-related proteins in response to exogenous administration of NO. This family of proteins is critical to actin elongation, powering the expansile apparatuses of cells, the filopodia and lamellapodia, (27 and recently rev. in 28) and is regulated by NO (29). Immunohistochemistry of VASP protein was performed on cultured EPCs. Incubation of the EPCs for 24 h with increasing concentration of NO resulted in increased VASP expression (Fig. 8) and a redistribution of this critical protein to filopodia. VASP, in the absence of NO, was distributed evenly throughout the cell. Whereas, with NO stimulation, VASP was upregulated and redistributed to the leading edge of the advancing cell processes. In addition, fluorescence-activated cell sorter analysis demonstrated that NO significantly increased expression of phospho-VASP in EPCs (Fig. 8D).

## DISCUSSION

The major finding of this report is that diabetic CD34<sup>+</sup> cells have markedly decreased migratory characteristics to multiple different stimuli that can be reversed by exogenous administration of physiologic concentrations of NO. The defect in migration in response to cytokines and growth factors may be responsible for the marked atherosclerosis, peripheral vascular disease, and delayed wound healing typically seen in diabetic patients. The diabetic CD34<sup>+</sup> cells were also rigid compared with those cells isolated from healthy control subjects. Like migration, treatment with NO induced deformability of the diabetic CD34<sup>+</sup> cells. This suggested that NO was affecting the cytoskeletal structure of the CD34<sup>+</sup> cells. This was confirmed by NO causing a redistribution of the cytoskeletal proteins VASP and mammalian-enabled distribution in EPCs.

To fully investigate the nature of the migration defect of CD34<sup>+</sup> cells isolated from diabetic patients, we investigated the activity of CD26/dipeptidyl peptidase IV on the surface of these cells. This enzyme cleaves SDF-1 (13,15,16) and could potentially modulate EPC behavior. The CD34<sup>+</sup> cell defect in migration was associated with a marked increase in CD26/dipeptidyl peptidase IV activity in some diabetic patients. However, while increased cell surface cleavage of SDF-1 may potentiate the migration defect, our studies suggest that it is not the only mechanism. Diabetic patients with levels of dipeptidyl peptidase IV activity below those of healthy control subjects or whose dipeptidyl peptidase IV activity was inhibited by diprotein A still had diminished cell migration.

Since the diabetic CD34<sup>+</sup> cells did not migrate to VEGF or SDF-1 and diabetic white blood cells have been shown



**FIG. 8.** Redistribution of VASP and mammalian-enabled distribution in response to incubation with a NO donor. EPCs after 1 week of culture were treated with 100  $\mu\text{mol/l}$  NO-depleted DETA/NO (A) or 100  $\mu\text{mol/l}$  DETA/NO (B) for 24 h before fixation and immunohistochemistry. A: Control VASP immunohistochemistry of EPCs (after treatment with depleted DETA/NO) filopodia (white arrow) demonstrating minimal staining with VASP antibody. B: NO-induced upregulation and redistribution of VASP to filopodia (white arrow) in EPCs. C: Secondary antibody control for EPC immunohistochemistry. D: Percent increase in phospho-VASP-expressing cells after treatment with DETA/NO for the indicated amount of time as determined by fluorescence-activated cell sorter. Shown is the average of three separate experiments. Error bars  $\pm$  SE. \* $P < 0.05$  compared with the no DETA/NO-treated cells.

to be less deformable than cells from normal control subjects (14), we used the micropipette technique to study the intrinsic cytoskeletal structure. Cells isolated from patients with diabetes complications were so rigid that they could not even be suctioned into the micropipette. We found that 100% of the diabetic cells were not deformable as opposed to only 8.3% of control cells. However, after 24 h exposure with an NO donor, the CD34+ cells from diabetic patients demonstrated the same deformability as normal control subjects.

To determine the mechanism of the increased rigidity of the diabetic CD34+ cells, we studied the localization of VASP by immunohistochemistry; this family of proteins is critical for actin filament elongation powering the advancement of the leading edge of cells (28). In addition, NO has been shown to regulate VASP (29). In response to physiologic concentrations of NO, VASP immunolocalization was altered, VASP protein expression was increased, VASP was concentrated in the filopodia, and VASP phosphorylation was markedly increased. These changes are conducive to cell motility and are consistent with recently published observations concerning the role of NO in inducing long-lasting potentiation of hippocampal synapses by altering neuronal puncta via VASP (30).

There could be additional mechanisms by which NO could correct the diabetic CD34+ cell migration defect. NO is required for proper motility of endothelial cells (31). Diabetes, by affecting Akt phosphorylation, results in diminished NO generation. Exogenous administration of NO would bypass the endogenous need for AKT phosphorylation.

Alternatively, NO may be acting as a reactive oxygen scavenger. Reactive oxygen species, known to be markedly increased in diabetes and associated with complications (32,33), interfere with growth factor signaling (34–36). Thus, NO, by quenching reactive oxygen species, could possibly enhance VEGF and SDF-1 downstream receptor signaling. Specifically,  $\text{H}_2\text{O}_2$  has previously been shown to induce stress fibers in endothelial cells, inhibit-

ing normal motility. NO, by scavenging super oxide and indirectly reducing  $\text{H}_2\text{O}_2$  levels, could prevent  $\text{H}_2\text{O}_2$  induction of cytoskeletal reorganization via calcium/calmodulin-dependent protein kinase II, Janus kinase 2 and mitogen-activated protein kinase, and extracellular signal-related kinase (37). However, we believe this is less likely since the reactive oxygen scavenger uric acid could not fully correct the migration defect in CD34+ cells (data not shown).

Defects in EPC migration would result in decreased reendothelialization. This defect could extend to the micro- and macrovascular complications seen in diabetic patients. Diabetic eye disease is characterized by aberrant microvascular changes with insufficient intraretinal vascularization and excessive “preretinal” neovascularization. The decreased response of diabetic CD34+ cells to picomolar concentrations of SDF-1 as well as other growth factors may inhibit the ability of these cells to repair early capillary endothelial injury. If this injury is not repaired, acellular capillaries could result. With continued ischemic injury, the retina continues to produce SDF-1 and VEGF that accumulate in the vitreous. With the vitreous acting as a growth factor “sink,” increasing growth factor concentrations may ultimately be able to overcome the relatively unresponsive CD34+ cells in the diabetic individual. However, the concentrated SDF-1 and VEGF in the vitreous directs EPC and endothelial cell migration to the surface of the retina, leading to preretinal vascular pathology. Correcting the CD34+ cell migratory defect, before the acellular capillary stage, may result in early capillary repair, preventing the late accumulation of vitreal SDF-1 and classic, preretinal, diabetic proliferation.

Rapid reendothelialization, mediated by CD34+ cells, at the macrovascular level can reduce atherosclerosis, peripheral vascular disease, and restenosis. These macrovascular complications are thought to be the result of endothelial injury (38,39) with possible inadequate endothelial repair and are all accelerated in the diabetic individual.

CD34+ cells, from healthy volunteers, significantly increase revascularization when injected into an ischemic hind limb of hypoinsulinemic diabetic mice (40) and accelerates revascularization and healing of full thickness skin wounds (41). Interestingly, injection of CD34+ cells in an ischemic limb of a normal mouse has no effect (40). This constellation of results suggests that diabetes has a detrimental effect on bone marrow and EPC homeostasis. Other conditions such as ischemia, smoking, and increased cholesterol have also been shown to have injurious effects on EPC number and function (42,43).

Administration of migratory CD34+ cells may represent an easily accessible and safe therapeutic approach to correct the multitude of vascular abnormalities seen in patients with diabetes. However these results should serve as a caution to in vivo studies using CD34+ cells as a therapeutic modality; improvement of CD34+ cell function in diabetic patients without simultaneously decreasing the levels of SDF-1 and other growth factors in the vitreous could result in worsening preretinal neovascularization. This is especially clinically relevant given the development of dipeptidyl peptidase IV inhibitors as an indirect means to stimulate insulin secretion and decrease glucagon secretion (44,45). These inhibitors by inhibiting SDF-1 degradation could potentiate CD34+ cell migration, and while this may slightly improve endothelial repair, it could worsen preretinal neovascularization, especially if given in the absence of decreasing the concentration of SDF-1 within the vitreous.

In conclusion, we have demonstrated that CD34+ cell dysfunction in diabetes is the result of alterations in the EPC cytoskeleton. The defect in SDF-1-induced migration seen in diabetic patients with end-organ damage is reversed by physiologic NO concentrations. We demonstrate that NO treatment improves deformability and normalizes the migration of diabetic cells likely by leading to phosphorylation and redistribution of VASP to the advancing edge, directly enhancing cell motility.

#### ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Grant DK02537, Gatorade Research Funds, a Baxter Renal Discovery Grant to M.S.S., and the Juvenile Diabetes Research Foundation International and National Institutes of Health Grants EY012601, EY007739, and EY14818 to M.B.G.

The authors thank Dr. G.C. Finlayson for help recruiting patients, Dr. David A. Antonetti for helpful suggestions regarding experimental design and critical review of the manuscript, and Dr. Daniel Purich for discussions concerning VASP.

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