Growth factors/chemokines in diabetic vitreous and aqueous alter the function of bone marrow-derived progenitor (CD34+ ) cells in humans

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Balaiya S, Grant MB, Priluck J, Chalam KV. Growth factors/chemokines in diabetic vitreous and aqueous alter the function of bone marrow-derived progenitor (CD34+) cells in humans. Am J Physiol Endocrinol Metab 307: E695–E702, 2014. First published August 26, 2014; doi:10.1152/ajpendo.00253.2014.—Ocular ischemic microenvironment plays a critical role in the progression of diabetic retinopathy (DR). In this study, we investigated the effect of vitreous and aqueous obtained from proliferative DR patients on the function of CD34+ cells derived from healthy humans. Human CD34+ cells were incubated with vitreous or aqueous of subjects with PDR. After incubation, cell migration of CD34+ was evaluated with CXCL12. Intracellular levels of nitric oxide (NO) were measured with DAF-FM. Tube formation assay was used to evaluate the effect of treated CD34+ cells on in vitro angiogenesis. Angiogenic protein array and mass spectrometry (MS) were performed to ascertain the factors secreted by healthy nondiabetic CD34+ cells exposed to diabetic vitreous or aqueous. PDR vitreous/aqueous reduced migration of CD34+ cells (672.45 ± 42.1/376.75 ± 101.7 AU; P < 0.01) and attenuated intracellular NO levels (182 ± 1.4/184.5 ± 6.3 AU, P = 0.002). Pretreatment with PDR vitreous suppressed tube formation of human retinal endothelial cells (64 ± 1.6 vs. 80 ± 2.5). CD34+ exposed to PDR vitreous resulted in the increased expression of CXCL4 and serpin F1, whereas CD34+ exposed to PDR aqueous showed increased expression of CXCL4, serpin F1, and endothelin-1 (ET-1). MS analysis of CD34+ (exposed to PDR vitreous) expressed J56 gene segment, isoform 2 of SPARC-related modular calcium-binding protein 2, isoform 1 of uncharacterized protein c1 orf167, integrin α-M, and 40s ribosomal protein s21. Exposure of healthy nondiabetic CD34+ cells to PDR vitreous and aqueous resulted in decreased migration, reduced generation of NO, and altered paracrine secretory function. Our results suggest that the contribution of CD34+ cells to the aberrant neovascularization observed in PDR is driven more by the proangiogenic effects of the retinal cells rather than the influence of the vitreous.

CD34+; progenitor cells; vitreous; aqueous; proliferative diabetic retinopathy

PROLIFERATIVE DIABETIC RETINOPATHY (PDR), the leading cause of blindness in working adults, affects 50% of individuals with 20 yr of having diabetes (29). PDR is associated with characteristic pathological changes such as microvascular injury, chronic inflammation, edema, neurodegeneration, and angiogenesis, secondary to retinal hypoxia from nonperfusion (8, 20).

Hypoxia releases several angiogenic growth factors/chemokines, including VEGF and stromal-derived factor-1 (SDF-1), that attract CD34+ cells (progenitor cells) to the sites of injury (20). These bone marrow-derived progenitors participate in repair and facilitate the restoration of vascular homeostasis in ischemic tissues (17, 32, 42). However, a decrease in the number and dysfunction of circulating CD34+ cells has been observed in diabetic retinopathy, nephropathy, and coronary artery disease (14, 27, 37, 46, 51). Vitreous injection of human CD34+ cells from diabetic individuals with microvascular complications showed impaired function and inability to repair injured retinal vasculature in diabetic rodents (6). CD34+ cell dysfunction occurs in part as a result of altered vasodilator-stimulated phosphoprotein (VASP) phosphorylation in diabetes, a requisite for vascular endothelial cell motility (1, 37, 52).

Although the number of CD34+ cells is altered in PDR (25), their possible role in the pathogenesis of proliferative diabetic vascular retinopathy in humans remains largely unknown. In this study, we investigated the effect of cytokines/proteins present in ocular fluids of PDR patients on the ability of CD34+ cells from healthy subjects to migrate, generate nitric oxide, and modulate in vitro angiogenesis.

METHODS

Characteristics of subjects. The study protocol was reviewed and approved by the Institutional Review Board at the University of Florida (UFJ 2012-91), Jacksonville. Twenty-two subjects, 11 with PDR and 11 controls, were recruited, and consent was obtained for participation in this study. Based on Early Treatment of Diabetic Retinopathy Study classification, PDR was defined as subjects with small or large neovascularization on the optic disc or elsewhere and vitreous hemorrhage with retinal neovascularization. Subjects with PDR who underwent vitrectomy at the Department of Ophthalmology, University of Florida, Jacksonville, were selected for the study. Subjects who underwent vitrectomy for epimacular membrane or macular hole served as controls. The following exclusion criteria for both study groups were used: evidence of ongoing acute or chronic infection (HIV, hepatitis B or C, tuberculosis), history or slit-lamp evidence of ocular trauma, prior intraocular surgery, prior surgery or pan-retinal photocoagulation, and use of systemic antimetabolites and immunosuppressants. PDR subjects had a mean age of 58.5 ± 14.3 yr compared with 66.8 ± 4.6 yr in control subjects.

Aqueous humor collection. Collection of aqueous humor was performed by a retinal surgeon (K. V. Chalam). Briefly, operated eyes were anesthetized with topical 1% tropicaine gel solution. The eye was prepped with 5% providine iodine solution and draped for surgery in the usual sterile manner. Aqueous humor sample was collected under the surgical microscope using a 1-ml tuberculin syringe and a 30-gauge needle to avoid damage to the irid and the anterior lens capsule and to prevent protein contamination. The aqueous humor samples were rapidly cooled on ice, centrifuged to remove cells, and immediately stored in a −80°C freezer.

Vitreous sample collection. Vitreous samples were collected during the vitrectomy procedure. In brief, the operated eye was anesthetized, prepped with 5% providine iodine solution, and draped for surgery in the usual sterile manner. A 25-gauge trochar was inserted into the standard position for the three-port parsplana vitrectomy. The vitrec-
tor was introduced, and with active cutting 0.6 ml of vitreous was aspirated into a 1-ml tuberculin syringe prior to initiation of infusion. The vitreous samples were rapidly cooled on ice and immediately stored in a −80°C freezer.

Preparation of CD34+ cells. Mobilized human CD34+ cells (Lanza, Walkersville, MD) were used immediately following isolation or maintained in an undifferentiated state as per the manufacturer’s protocol. Briefly, isolated CD34+ cells were cultured using undifferentiated stem span medium (Stem Cell Technologies, Vancouver, BC, Canada) supplemented with cytokine cocktail containing interleukin-3, interleukin-6, Flt ligand, and stem cell factor (Stem Cell Technologies). CD34+ cells (10,000 cells/well) were plated in a 96-well plate and suspended in 100 µl of Ham’s F-12 medium (Cellgro, Manassas, VA) with 5% fetal bovine serum, whereas the negative control group received only Ham’s F-12 medium. Cells were allowed to migrate for 16 h at 37°C at 5% CO2. A diluted fluorescent dye (CyQuant GR Dye; Millipore) with lysis buffer that lyzes the cells and binds to the cellular nucleic acids was added to lower wells. The concentration of the fluorescent dye was determined using a microplate reader (Synergy HT; Bio-Tek Instruments, Winooski, VT) with an excitation of 485 ± 20 nm and an emission of 528 ± 20 nm. The number of migrating cells was expressed in arbitrary fluorescent units (AFU). Experiments were run on triplicate wells for each sample and duplicated for concordance.

Migration of CD34+ cells using chemotaxis assay. CD34+ cells (10,000 cells/well) incubated either with control and PDR vitreous or aqueous samples at a concentration of 5% were loaded into the upper chamber of migration assay kit (Chemicon International, Millipore, Temecula, CA). Chemotaxiant CXCL12 (100 nmol/l; R & D Systems, Minneapolis, MN) in Ham’s F-12 medium was added to the lower chamber. The positive control group was treated with 20% fetal bovine serum, whereas the negative control group received only Ham’s F-12 medium. Cells were allowed to migrate for 16 h at 37°C at 5% CO2. A diluted fluorescent dye (CyQuant GR Dye; Millipore) with lysis buffer that lyzes the cells and binds to the cellular nucleic acids was added to lower wells. The concentration of the fluorescent dye was determined using a microplate reader (Synergy HT; Bio-Tek Instruments, Winooski, VT) with an excitation of 485 ± 20 nm and an emission of 528 ± 20 nm. The number of migrating cells was expressed in arbitrary fluorescent units (AFU). Experiments were run on triplicate wells for each sample and duplicated for concordance.

Determination of nitric oxide production by 4-amino-5-methylamino-2,7’-difuorofluorescein diacetate. Nitric oxide (NO) production was quantified in CD34+ cells using NO-sensitive 4-amino-5-methylamino-2,7’-difluorofluorescein diacetate (DAF-FM; Invitrogen, Carlsbad, CA), as described previously (22). In brief, CD34+ cells (2,000 cells/well; 100 µl of Ham’s F-12 medium with 10% or PDR/control aqueous or vitreous) on a 96-well plate were loaded with DAF-FM diacetate (10 µM) for 45–60 min in Hanks’ balanced saline solution (Invitrogen). The fluorescence of DAF-FM-loaded cells was detected at excitation/emission maxima of 495/515 nm using a microplate reader (Synergy HT) and observed using an inverted-phase contrast microscope with fluorescence filter (Olympus IX51; Olympus, Center valley, PA). Changes in DAF fluorescence were expressed in AFU compared with time or vehicle control, i.e., cells that received no treatments but were loaded with DAF-FM.

Matrigel angiogenesis assay in vitro. Human retinal microvascular endothelial cells (HREC; Angio-Protemic, Boston, MA) were cultured in endothelial growth medium (Angio-Protemic) at 37°C in logarithmic scale in a 75-cm2 cell culture flask in an incubator under standard conditions of 95% air and 5% CO2. Matrigel (growth factor reduced; BD Biosciences, San Jose, CA) was thawed overnight at 4°C, and 30 µl of matrigel was added to each well of a 96-well plate and polymerized at 37°C for 30 min before use.

HRECs (10,000 cells/well) were seeded on a 96-well plate that was previously coated with matrigel and placed in humidified atmosphere of 5% CO2 at 37°C after the addition of vitreous- or aqueous-exposed CD34+ cells (2,000 cells/well). HRECs placed in medium containing either vitreous or aqueous, but without CD34+ cells, were also analyzed. HRECs placed in medium containing 10% serum served as internal controls. After 16 h, tube-like structures were observed, using an inverted-phase contrast bright-field microscope at ×10 magnification (Olympus IX 51), as described previously (18). Five fields per well were chosen randomly, and the number of tubes was counted and presented. Experiments were run on triplicate wells for each sample and duplicated for concordance.

Angiogenic protein array. The presence of angiogenic proteins was tested in vitreous or aqueous from controls, controls incubated with CD34+ cells, vitreous or aqueous from PDR, and CD34+ incubated in PDR vitreous or aqueous, using the human angiogenesis antibody array kit (R & D Systems) as recommended by the manufacturer. In brief, nitrocellulose membranes with coated antibodies in duplicate for each protein spot were blocked for 1 h at room temperature. After blocking, membranes were washed and incubated overnight at 2–8°C with the test sample and the detection antibody. Excess antibodies were washed from the membrane, and the membranes were probed with streptavidin-horseradish peroxidase for 30 min at room temperature. Immunoreactive spots were detected using enhanced chemiluminescence. Array data were quantified by measuring the sum of the intensities within the spot area using ImageJ analysis software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html) and expressed in arbitrary units (AU).

Protein identification by liquid chromatography-mass spectrometry/mass spectrometry: sample preparation and in-solution digestion. Pooled samples from each of the vitreous or aqueous from controls, controls incubated with CD34+ cells, vitreous or aqueous from PDR, and CD34+ incubated in PDR vitreous or aqueous were desalted, and was buffer exchanged with 50 mM ammonium bicarbonate using a 3-kDa cutoff spin column (Millipore). Desalted samples were denatured and reduced using diithiothreitol and loaded with iodoacetic acid for alkylation at 25°C/45 min. Alkylated samples were digested by endoproteases overnight at 37°C.

The enzymatically digested samples were further purified by desalting using a capillary trap (LC Packings PepMap) and loaded onto an LC Packing C18 Pep Map nanoflow HPLC column to remove residual buffers and detergents. Purified peptide samples were loaded onto an LTQ Orbitrap XL mass spectrometer (ThermoFisher Scientific, West Palm Beach, FL) for liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) analysis. The ion spray voltage was set to 2,200 V, and full MS scans were acquired with a resolution of 60,000 in the orbitrap from 300 to 2,000 m/z. The five most intense ions were fragmented by collision-induced dissociation.

Dynamic exclusion was set to 60 s. Protein search algorithm. All MS/MS samples were analyzed using Mascot (version 2.2.2; Matrix Science, London, UK). Mascot was searched with a fragment ion mass tolerance of 0.8 Da and a parent ion tolerance of 10 ppm. Iodoacetamide derivative of cysteine, deamidation of asparagine and glutamine, and oxidation of methionine are specified in mascot as variable modifications. Scaffold (version 3.6; Proteome Software, Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted at >80.0% probability.

Statistical analysis. Data are expressed as means ± SD or SE. Unpaired two-tailed Student’s t-test or one-way ANOVA was used to determine statistical significance. All statistical analyses were performed using GraphPad Prism 6 (GraphPad, La Jolla, CA), with a P value of <0.05 considered to be significant. Corresponding significance levels are indicated in the figures.

RESULTS

Diabetic vitreous and aqueous inhibits migration of CD34+ cells. Vascular regeneration and angiogenesis require migration of various cells. To examine the effects of PDR vitreous or aqueous, healthy human CD34+ cells were incubated with either PDR or control vitreous or aqueous. CD34+ cells showed a significantly reduced migratory response (67.45 ± 42.1 AFU, P = 0.0009) to CXCL12 when they were pretreated with PDR vitreous (16 h) compared with pretreatment with control vitreous (16 h, 794.8 ± 36.6 AFU; Fig. 1A). Similarly, the migratory response in cells treated with PDR aqueous was...
migratory responses in the positive and negative controls were cells treated with control aqueous (P < 0.001). Compared with untreated CD34 cells treated with 5% PDR/control vitreous or aqueous and maintained at 37°C for 17 h to evaluate migration. A: migration of CD34+ cells treated with 5% PDR or control vitreous; P < 0.01; B: migration of CD34+ cells treated with 5% PDR or control aqueous (P < 0.05); x-axis represents treatment condition, and y-axis represents migration of CD34+ cells in arbitrary fluorescent units (AFU). *P = 0.0009 and **P = 0.01, significant difference compared with corresponding control. Data were analyzed by Student’s t-test and represented as error bars ± SE (n = 3).

Intracellular NO decreased in CD34+ cells treated with vitreous and aqueous from subjects with PDR. In CD34+ cells, intracellular NO levels (in response to PDR vitreous or aqueous treatment) were determined using NO-sensitive DAF-FM (Fig. 2). Compared with untreated CD34+ cells (276.5 ± 12.0 AFU), cells that were treated with PDR vitreous showed reduced NO generation (182 ± 1.4 AFU), whereas cells treated with PDR aqueous showed 184.5 ± 6.3 AFU. The reduction in bioavailable NO levels was highly significant (P = 0.002; Fig. 2D). CD34+ cells that were treated with either control vitreous (281.5 ± 3.5 AFU) or aqueous (284.7 ± 6.1 AFU) showed higher NO levels than untreated CD34+ cells (Fig. 2E).

In vitro angiogenesis assay. In vitro tube formation assay was used to determine whether exposure of CD34+ cells to the aqueous or vitreous would influence their interaction with HREC (Fig. 3). After overnight incubation, HREC treated with control vitreous showed 80 ± 2.5 tube-like structures. PDR vitreous-treated HREC showed 64 ± 1.6 tube-like structures (P = 0.04; Fig. 3G). Treatment with PDR aqueous caused a similar decrease in tubular network formation (60 ± 1.8) compared with 81 ± 2.2 tubes present in control aqueous-treated HREC (P = 0.011; Fig. 3H). We found a significantly decreased number of tubes in PDR vitreous- or aqueous-exposed HREC compared with con-

736.75 ± 101.7 AFU compared with 876.3 ± 70.9 AFU in cells treated with control aqueous (P = 0.01; Fig. 1B). The migratory responses in the positive and negative controls were 2,034.6 ± 303.9 and 221.3 ± 24.2 AFU, respectively.

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control vitreous- or aqueous-exposed HREC \((P < 0.05; \text{Fig. 3, I and J})\).

**Angiogenic protein array.** The major function of CD34\(^+\) cells is to provide paracrine support to the resident vasculature. To characterize the influence of PDR vitreous on the paracrine function of these cells, angiogenic protein expression was measured and compared with cells exposed to control vitreous (Fig. 4). Two proteins, CXCL4 (platelet factor 4) and serpin peptidase inhibitor clade F (Serpin F1), were increased significantly in the supernatants of PDR vitreous-treated CD34\(^+\) cells \((6,740\) and \(2,299.4\) AU, respectively; \text{Fig. 4B}) compared with controls \((1,408\) and \(1,087.6\) AU respectively, \(P = 0.0001\) and \(P = 0.01\)). Thrombospondin-1 (TSP-1; \(176\) AU), dipeptidyl peptidase IV [DPP IV (CD26); \(301\) AU], and angiopoietin-2 (Ang-2; \(518.2\) AU) were expressed only in the supernatants of CD34\(^+\) cells treated with PDR vitreous and at the lower level in cells treated with control vitreous. However, other proteins known to be altered in diabetes, such as endothelin-1 (ET-1) and tissue inhibitor of metalloproteinase-1 (TIMP-1), were expressed equally in supernatants of CD34\(^+\) cells exposed to either control \((251.4\) and \(122.9\) AU, respectively) or PDR vitreous-treated groups \((273.9\) and \(198.9\) AU, respectively; \text{Fig. 4B}) Except for serpin F1, endothelin-1, and TIMP-1, we did not observe any of these proteins in control or PDR vitreous alone (not incubated with CD34\(^+\); Fig. 4C).

ET-1 and serpin F1 were increased significantly in the supernatants of PDR aqueous-treated CD34\(^+\) cells \((2,081.40\) and \(4,362.62\) AU, respectively) compared with controls \((955.0\) and \(3,102.64\) AU, respectively, \(P < 0.05; \text{Fig. 4D})\). CXCL4 was expressed only in the supernatants of CD34\(^+\) cells treated with PDR aqueous \((2,807.96\) AU) and at the lower level in cells treated with control aqueous \((21.24\) AU). Unlike serpin F1 and endothelin-1, we did not observe CXCL4 in control or PDR aqueous (not incubated with CD34\(^+\); \text{Fig. 4E}).

**Protein identification by LC-MS/MS.** We next examined the protein expression of CD34\(^+\) cells exposed to either PDR or control vitreous. MS analysis revealed the presence of five proteins specific to PDR vitreous-treated CD34\(^+\) cells, such as J66 gene segment, isofrom 2 of secreted protein acidic and rich in cysteine-related modular calcium-binding protein 2, isofrom 1 of cysteine-related modular calcium-binding protein 2, and cysteine-related modular calcium-binding protein 2.
uncharacterized protein c1 orf167, integrin α-M, and 40s ribosomal protein s21. We did not observe any of these proteins in control or PDR vitreous (not incubated with CD34+).

The following 10 proteins were observed in both control and PDR aqueous-treated CD34+ cells compared with proteins observed in control and PDR aqueous without exposure to CD34+: integrin α-M, haptoglobin isoform 2 preproprotein, putative uncharacterized protein, PRO2275, uncharacterized protein, isoform 1 of α-1B-glycoprotein, complement factor 1, isoform 1 of coiled-coil domain-containing protein 73, leukemia inhibitory factor receptor, and uncharacterized protein C9orf104. However, four proteins were expressed only in PDR
aqueous-treated CD34\(^+\) cells: isoform 1 of 1-phosphatidylinositol-3-phosphate 5-kinase, M-phase inducer phosphatase 3, α-2,8-sialyltransferase 8E, and isoform 1 of G protein-regulated inducer of neurite outgrowth 1.

**DISCUSSION**

CD34\(^+\) isolated from diabetics has exhibited reduced migration and altered endothelial nitric oxide synthase expression in both human and animal studies (47). However, the impact of the diabetic ocular environment on human CD34\(^+\) cell function has not been examined previously. To begin to address this, we tested the impact of PDR vitreous or aqueous humor on the function of healthy CD34\(^+\) cells. Specifically, we investigated migratory function and NO generation by CD34\(^+\) cells after exposure to PDR vitreous or aqueous. Migratory deficits have been associated with peripheral vascular disease, delayed wound healing, and atherosclerosis in diabetic patients (37), and reduced migration of these cells in vascular disease states has been well characterized. However, the effect of vitreous and aqueous from diseased individuals on healthy CD34\(^+\) cells has not been studied.

In diabetes, the reduction in NO occurs in part because of altered Akt phosphorylation (3). Decreased bioavailable NO increases the rigidity of diabetic CD34\(^+\) cells due to altered phosphorylation of cytoskeletal protein VASP. The VASP family of proteins is critical for actin filament elongation, which is in turn required for the advancement of the leading family of proteins is critical for actin filament elongation, phosphorylation of cytoskeletal protein VASP. The VASP on the function of healthy CD34\(^+\) cells. Specifically, we investigated migratory function and NO generation by CD34\(^+\) cells after exposure to PDR vitreous or aqueous. Migratory deficits have been associated with peripheral vascular disease, delayed wound healing, and atherosclerosis in diabetic patients (37), and reduced migration of these cells in vascular disease states has been well characterized. However, the effect of vitreous and aqueous from diseased individuals on healthy CD34\(^+\) cells has not been studied.

In diabetes, the reduction in NO occurs in part because of altered Akt phosphorylation (3). Decreased bioavailable NO increases the rigidity of diabetic CD34\(^+\) cells due to altered phosphorylation of cytoskeletal protein VASP. The VASP family of proteins is critical for actin filament elongation, which is in turn required for the advancement of the leading edge of migrating cells (1, 24). Reduced migration of CD34\(^+\) cells and the decreased levels of NO shown in this study suggest that the PDR vitreous and aqueous has direct deleterious effects on healthy CD34\(^+\) cells.

We examined the angiogenic potential of PDR vitreous and aqueous using the well-established tube formation assay by coculturing of CD34\(^+\) cells exposed to PDR vitreous or aqueous and HRECs (38). A substantial decrease in the number of tubes was detected. We postulated that this was likely due to alterations of secreted factors from the CD34\(^+\) cells treated with aqueous or vitreous.

Circulating CD34\(^+\) cells arise from the bone marrow, migrate into the bloodstream, and home to peripheral vascular beds such as retina or kidney, repairing injured vasculature by providing paracrine support to the resident vasculature (28). It is well accepted that the main function of CD34\(^+\) cells is to provide paracrine support to injured vasculature rather than their differentiation to endothelial cells, and in diabetes this function is altered (20).

To assess the impact of PDR vitreous and aqueous on the paracrine secretion of CD34\(^+\) cells, we performed both angiogenic arrays and proteomic analysis using LC-MS/MS. Seven cytokines that have previously been shown to play a vital role in autocrine CD34\(^+\) cell function were observed in our study.

CXCL4 or platelet factor 4, an abundant platelet α-granule protein, has chemotactic and functional priming activity on neutrophils (34, 43). Thus the production of CXCL4 by CD34\(^+\) cells exposed to PDR vitreous or aqueous could promote retinal inflammation by recruiting neutrophils from the circulation to the retina, promoting pathological inflammation. CXCL4 has been reported to inhibit proliferation of early committed hematopoietic progenitors at concentrations in the low nanomolar range in vitro (10) but requires micromolar concentrations in vivo. Adhesion to CXCL4 causes cytoskeletal rearrangement in CD34\(^+\) hematopoietic cells (11) and regulates migration.

Serpin F1 (serpin peptidase inhibitor clade F) inhibits the migration of endothelial cells in a dose-dependent manner and mediates apoptosis through the p38 MAPK or Fas/Fasl pathway (7, 30, 31, 44). Recent reports noted increased serpin F1 in the vitreous of PDR subjects (2, 12). Consistent with these findings, the observation of increased expression of serpin F1 in CD34\(^+\) cells treated with PDR vitreous or aqueous could enhance their dysfunction.

An inverse association between TSP-1 expression and adhesion activity in CD34\(^+\) cells has been shown (26). Recent evidence shows that TSP-1 is a multifunctional protein that interacts with a number of matrix proteins and cell surface receptors and represents an important link between diabetes, high glucose levels, and accelerated development of atherosclerotic lesions in diabetes (39, 41). TSP-1 has specific binding sites to fibronectin. Mature endothelial cells have high affinity to the fibronectin/TSP-1 complex via α8β1 integrin on the endothelial cell surface (36). Whether TSP-1 impacts CD34\(^+\) cells in this manner remains to be determined.

CD26 (DPP IV) is a membrane-bound extracellular peptidase involved in cell membrane-associated activation of intracellular signal transduction pathways, cell-cell interaction, and enzymatic activity exhibited by both the membrane-anchored and the soluble forms of the enzyme. It is expressed constitutively on many hematopoietic cell populations, including T-lymphocytes, endothelial cells, fibroblasts, and epithelial cells (9). In addition, CD26/DPP IV is present in a catalytically active soluble form in plasma. CD26/DPP IV downregulates CXCR12 activation of CXCR4 receptor-presenting cells by cleaving the NH2-terminal dipeptide of CXCL12/SDF-1α. NH2-terminally truncated CXCL12 (without the first 2 amino acids) lacks chemotactic activity (45). CXCL12 is a critical chemoattractant for human CD34\(^+\) cells and stem/progenitor cell populations, and it is considered an important component of the migration, homing, and mobilization of these progenitor cells. CD34\(^+\) cells isolated from diabetic patients demonstrate a marked defect in migration to SDF-1. This defect was associated, in some but not all patients, with increased cell surface activity of CD26/DPP IV (37). Inhibition of CD26 in type 2 diabetic patients showed a significant increase in CD34\(^+\) cell function after 4 wk and a decrease in monocyte chemoattractant protein-1 (15). Pharmacological inhibition of CD26 increased myocardial homing of circulating CXCR4\(^+\) stem cells, reduced cardiac remodeling, and improved heart function and survival (50). Thus, increased CD26 expression would have an adverse effect on CD34\(^+\) cell responsiveness to CXCL12.

Angiopoietins have potentially complex direct and indirect effects on inflammatory responses. Ang-2 destabilizes endothelial cell-cell junctions and enhances leakage of inflammatory cells and also sensitizes endothelial cells to TNFα (16, 33). Glucose, hypoxia, adrenocorticotropic, and TNFα upregulate Ang-2 expression, and Sonic hedgehog, a secreted growth factor, upregulates expression of both Ang-1 and Ang-2 (19, 23, 35, 48, 49). However, the effects of Ang-2 on CD34\(^+\) cells remain to be determined.

We also observed an increase in the levels of TIMP-1, a glycoprotein that controls the activity of matrix and other metalloproteinases in CD34\(^+\) cells exposed to PDR vitreous.
TIMP-1 promotes the proliferation of human progenitor cells and has been shown to regulate cell growth, apoptosis, and differentiation that are independent of its metalloproteinase inhibitory activity (5, 13, 40). ET-1, a potent vasoconstrictor peptide in endothelial cells, stimulates a number of biological actions, including vasoconstriction, proinflammatory actions, mitogenic and proliferative effects, and formation of free radicals. ET-1 is associated with hypertension and vascular inflammation and suggested as a marker of endothelial dysfunction and has been associated with circulating endothelial progenitor cell mobilization in type 2 diabetic patients (4, 21). The increase in ET-1 in CD34+ cells following exposure to PDR aqueous suggests a stress response in these cells.

MS leads to identification of both nuclear and intracellular proteins. We found that nutrition transport, inflammation, and signal transduction pathway proteins were increased in CD34+ cells exposed to PDR compared with control vitreous or aqueous. The reported increase in phosphorylidyinositol 3-kinase (PI3K) protein expression in CD34+ cells exposed to PDR vitreous supports an earlier report suggesting that activation of CD34+ to CXCL12 involves the CXCR4/G, protein/PI3K/Akt pathway (3).

In summary, exposure of healthy nondiabetic CD34+ cells to PDR vitreous or aqueous resulted in decreased migration, reduced generation of NO, and altered paracrine secretory function. The major findings of this study were unexpected, as we anticipated that the PDR vitreous would increase the migration, proliferation, and NO generation of healthy CD34+ cells as well as increase tube formation of the HRECs. In marked contrast, PDR vitreous did not have this effect. This suggests that the factors released by the retina rather than the CD34+ may play a more critical role in orchestrating the angiogenic response of the retinal endothelium to promote preretinal neovascularization. Considered another way, proangiogenic growth factor released by ischemic retina provides the intense stimulus to promote retinal neovascularization rather than the paracrine CD34+ cell secretome.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
S.B., M.B.G., and J.P. conception and design of research; S.B. performed experiments; S.B. analyzed data; S.B. interpreted results of experiments; S.B. prepared figures; S.B. drafted manuscript; S.B., M.B.G., and K.V.C. edited and revised manuscript; S.B., M.B.G., J.P., and K.V.C. approved final version of manuscript.

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