

Advanced Glycation of Fibronectin Impairs Vascular Repair by Endothelial Progenitor Cells: Implications for Vasodegeneration in Diabetic Retinopathy

Ashay D. Bhatwadekar,¹ Josephine V. Glenn,¹ Gang Li,² Tim M. Curtis,¹ Tom A. Gardiner,¹ and Alan W. Stitt¹

PURPOSE. Vascular repair by marrow-derived endothelial progenitor cells (EPCs) is impaired during diabetes, although the precise mechanism of this dysfunction remains unknown. The hypothesis for the study was that progressive basement membrane (BM) modification by advanced glycation end products (AGEs) contributes to impairment of EPC reparative function after diabetes-related endothelial injury.

METHODS. EPCs isolated from peripheral blood were characterized by immunocytochemistry and flow cytometry. EPC interactions on native or AGE-modified fibronectin (AGE-FN) were studied for attachment and spreading, whereas chemotaxis to SDF-1 was assessed with the Dunn chamber assay. In addition, photoreactive agent-treated monolayers of retinal microvascular endothelial cells (RMECs) produced circumscribed areas of apoptosis and the ability of EPCs to “endothelialize” these wounds was evaluated.

RESULTS. EPC attachment and spreading on AGE-FN was reduced compared with control cells ($P < 0.05$ – 0.01) but was significantly restored by pretreatment with Arg-Gly-Asp (RGD). Chemotaxis of EPCs was abolished on AGE-FN but was reversed by treatment with exogenous RGD. On wounded RMEC monolayers, EPCs showed clustering at the wound site, compared with untreated regions ($P < 0.001$); AGE-FN significantly reduced this targeting response ($P < 0.05$). RGD supplementation enhanced EPC incorporation in the monolayer, as determined by EPC participation in tight junction formation and restoration of transendothelial electric resistance (TEER).

CONCLUSIONS. AGE-modification of vascular substrates impairs EPC adhesion, spreading, and migration; and alteration of the RGD integrin recognition motif plays a key role in these responses. The presence of AGE adducts on BM compromises repair by EPC with implications for vasodegeneration during diabetic microvasculopathy. (*Invest Ophthalmol Vis Sci.* 2008; 49:1232–1241) DOI:10.1167/iovs.07-1015

Diabetic retinopathy (DR) is associated with progressive damage of the retinal microvascular endothelium eventually leading to capillary occlusion. The resultant ischemia has

serious implications for the retina and precipitates sight-threatening pre-retinal neovascularization and macular edema in a significant proportion of patients. The pathogenesis of DR is complex and hyperglycemia-induced retinal basement membrane (BM) thickening may play an important role in the progression of this complication. A high glucose level may induce excessive production of BM proteins such as fibronectin (FN)^{1,2} or collagen,^{3,4} which in turn can compromise endothelial migration and replication, precipitating delayed endothelial wound healing.⁵ Moreover, advanced glycation end product (AGE) modification of BM components induces detrimental effects on the diabetic retina by disrupting normal vascular cell function.^{6,7} AGE adducts on BM can lead to impaired cell-matrix interactions and growth factor depletion in endothelial cells^{8,9} and pericytes.^{10,11} This effect has a marked impact on pro-survival signaling in microvascular endothelial cells and ultimately leads to cell death by anoikis.¹²

Bone marrow-derived endothelial progenitor cells (EPCs) home to the sites of tissue injury or ischemia and make an important contribution to organ regeneration by promoting vascular integrity during wound repair.¹³ These cells are recruited to the pre-existing “damaged” vasculature via adhesion molecules and cytokine gradients,¹⁴ whereas subsequent differentiation and incorporation of EPCs into vessels is mediated by complex interactions with BM-associated signals.¹⁵ The BM component protein FN harbors crucial signals for EPC differentiation,¹⁵ and sequestration of growth factors within the FN substrate may provide synergism for progenitor differentiation, which may not occur with collagen IV, vitronectin, or fragments of FN.¹⁶ Accumulating evidence shows that healthy EPCs may respond to ischemic retina by contributing to retinal vessel development^{17,18} or pathogenic vessel formation.^{19,20} EPCs provide a circulating pool of endothelial cells for the repair of ischemic retina after diabetes-related vasodegeneration.²¹ The availability of these cells may be of particular importance in the case of damaged capillary endothelial cells which become less able to replace gaps in the monolayer,²² as they reach their so-called Hayflick limit.²³

Although introduction of autologous EPCs appear to contribute to capillary persistence in a nondiabetic model of retinal vascular attenuation,²⁰ EPCs derived from diabetic patients do not revascularize ischemic retina.¹⁸ Thus, for therapeutic purposes, it is necessary to introduce healthy EPCs. To this end, in a recent clinical trial, use of a heterogeneous population of EPCs was beneficial for treatment of patients with myocardial infarct.²⁴ Recent studies have shown that incubation of both early and late EPCs in high-glucose conditions impairs EPC function and proliferation²⁵; however, it remains unknown how the reparative properties of these endothelial progenitors and their incorporation within the retinal microvasculature is altered in the presence of AGEs. Pharmacologic approaches to correct EPC defects due to the diabetic milieu have been attempted²⁶; however, for efficient recruitment of these cells, ambient factors like BM modification and cytokine

From the ¹Centre for Vision Science and the ²Centre for Cancer Research and Cell Biology, Queen's University Belfast, Northern Ireland, United Kingdom.

Supported by INSIGHT, The Trust for the Visually Impaired, Guide Dogs for the Blind, Fight for Sight, and The Juvenile Diabetes Research Foundation (JDRF).

Submitted for publication August 7, 2007; revised October 4, 2007; accepted January 17, 2008.

Disclosure: **A.D. Bhatwadekar**, None; **J.V. Glenn**, None; **G. Li**, None; **T.M. Curtis**, None; **T.A. Gardiner**, None; **A.W. Stitt**, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked “advertisement” in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Alan W. Stitt, Centre for Vision Science, Queen's University Belfast, Royal Victoria Hospital, Grosvenor Road Belfast, Northern Ireland BT126 BA, UK; a.stitt@qub.ac.uk.

environment in the vicinity of malperfused vessels should be considered.

In the present study, we hypothesize that progressive BM modification by AGE-adduct accumulation during diabetic retinopathy could be the contributing factor in EPC incorporation and impaired vascular repair. Using *in vitro* approaches on a diabetes-like substrate, we assessed EPC dynamics that could offer a new insight into impact of AGE-modification on EPC adhesion and incorporation with implications for the pathogenesis of diabetic retinopathy.

METHODS

Isolation of Peripheral Blood Mononuclear Cell Fraction (hPBMCs) and EPC Culture

EPCs were isolated as described previously.²⁷ Briefly, fresh peripheral blood (not more than 8 hours postcollection) obtained from healthy volunteers was layered over a radiopaque contrast medium (Histopaque 1077; Sigma-Aldrich, Gillingham, UK). After centrifugation, the resultant mononuclear cell layer was washed twice with PBS (containing 2 mM EDTA) and resuspended in endothelial cell basal medium (EBM-2) supplemented with growth medium (EGM-2 MV SingleQuots; Cambrex Bio Science, Wokingham, Ltd., Berkshire, UK) containing 5% FBS, human VEGF, FGF-2, EGF, IGF-1, and ascorbic acid. The cells were then plated onto FN (Sigma-Aldrich)-coated plates (2 $\mu\text{g}/\text{cm}^2$) and the medium changed on days 4 and 7, after which time the plates showed numerous attached spindle-shaped cells (typically, 7–10 days).

Characterization of EPCs

The phenotype of EPCs was established by a range of assessments: Direct binding of FITC-conjugated *Ulex europaeus agglutinin* (UEA)-1 (Sigma-Aldrich) and uptake of 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine percholate (DiI)-labeled acetylated low-density lipoprotein (acLDL; Biomedical Technologies Inc., Stoughton, MA) were performed. For these assays, the cells were first incubated with acLDL DiI (10 $\mu\text{g}/\text{mL}$ in EBM-2 medium) at 37°C for 4 hours and later fixed with 1% paraformaldehyde and stained with UEA-1 (10 $\mu\text{g}/\text{mL}$ in 0.9% saline). For immunolocalization of CD31, the cells were incubated with CD31 primary antibody (Dako Cytomation, Ely, UK) followed by Alexa-Fluor 488 as the secondary antibody (Invitrogen-Molecular Probes, Eugene, OR).

After UEA-1 labeling, acLDL DiI uptake and CD31 immunolocalization, the cells were visualized with confocal microscopy. For quantitative characterization, the cells were incubated at 4°C for 30 minutes with FITC-labeled CD34 (Miltenyi Biotech, Surrey, UK) or CD14 (e-Bioscience, San Diego, CA) antibodies. In addition, the cells were stained for VEGFR2 and CD133 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) primary antibodies, followed by labeling with a secondary antibody conjugated to AlexaFluor 488 (Invitrogen-Molecular Probes). Flow cytometric analysis was performed (FACScalibur flow cytometer; BD Biosciences, Oxford, UK), and histograms showing the number of cells versus logarithmic fluorescence intensity were plotted. A minimum of 10,000 gated events per sample was used.

Preparation of AGE-Modified FN

Four-well chamber slides were coated with FN at 2 $\mu\text{g}/\text{cm}^2$ and incubated with methylglyoxal (MGO; Sigma-Aldrich) at 10, 50, or 100 μM or with PBS alone for 1 week at 37°C. After incubation, the slides were washed with PBS to remove MGO and unbound adducts. Under similar conditions, FN was incubated with MGO for the estimation of the MGO-derived AGE adducts argpyrimidine and carboxyethyl lysine (CEL) by HPLC and competitive AGE-ELISA, respectively. For argpyrimidine estimation the method outlined by Degenhardt et al.²⁸ was used. ELISA for CEL adducts was performed, using a monoclonal antibody that recognizes both CEL and carboxy methyl lysine (CML; kindly

provided by Suzanne Thorpe, University of South Carolina, Columbia). The ELISA was performed as previously described.²⁹

EPC Attachment and Spreading

EPCs were seeded on chamber slides coated with AGE-FN or native FN, as just described. After 3 hours, nonadherent cells were removed by washing the slides three times with PBS. Adherent cells were later stained with acLDL DiI and UEA-1 lectin, and dual-positive cells were counted in a minimum of 25 random fields using a 40 \times objective on an epifluorescence microscope. For spreading analysis, EPCs were seeded on native or AGE-FN and allowed to spread for 6 hours. The cells were then fixed and stained for filamentous actin (F-actin) using phalloidin (Invitrogen-Molecular Probes). EPCs were visualized using confocal microscopy, and spread or unspread cells were identified and subsequently quantified on the basis of their morphologic characteristics. As an indicator of focal adhesion kinase (FAK) signaling in EPCs, the cells were immunostained for phospho-FAK (tyrosine³⁹⁷) after application to native or AGE-FN for 12 hours. After brief fixation with 4% PFA, adherent EPCs were reacted with primary phospho-FAK (tyrosine³⁹⁷) antibody (Chemicon-Millipore, Temecula, MA) followed by secondary antibody incubation with AlexaFluor 488 (Invitrogen-Molecular Probes). Immunolocalization of phospho-FAK on the basal aspect of the spread cells was conducted by confocal microscopy. Immunoreactivity was quantified by counting hyperfluorescent spots using image-analysis software (ImageJ; National Institutes of Health, Bethesda, MD; available by ftp at zippy.nimh.nih.gov/ or at <http://rsb.info.nih.gov/nih-image/>; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD), as described previously.³⁰

EPC Chemotaxis

EPC chemotactic responses were assessed using the Dunn chamber assay, as previously described.³¹ Briefly, 24 hours before the experiment was conducted, EPCs were allowed to attach to coverslips coated with native or AGE-FN. The outer well of the Dunn chamber was filled with 50 ng/mL SDF-1 (R&D Systems Europe, Abingdon, UK), and the assembled Dunn chamber slide was maintained at 37°C on a heated stage of an inverted microscope. With a shutter control device, images were digitally recorded over the span of 10 hours, at intervals of 5 minutes. Data were analyzed (AQM 2001 software; Kinetic Imaging Ltd., Manchester, UK), and the speed and direction of EPC chemotaxis was further determined with a custom-designed computer program (Mathematica 3.0 software³¹) and shown as circular histograms. The speed of migration was expressed as the mean speed in micrometers per 10-minute interval.

RGD Modification and Supplementation of AGE FN with RGD

The ability of exogenous Arg-Gly-Asp (RGD; Sigma-Aldrich) tripeptide treatment to modulate EPC responses on AGE-FN was studied to determine whether supplementation of RGD attachment motifs "lost" by AGE-modification could modulate substrate interaction deficits. RGD peptide (1 mM) was immobilized on AGE-modified substrates by preincubation for 24 hours at 37°C, the plates were allowed to air dry and EPC attachment, spreading, migration, and expression of FAK were repeated in the presence or absence of RGD peptide using the same procedure as described earlier in the article.

In Vitro Model for Endothelial Cell Injury

Retinal microvascular endothelial cells (RMECs) were isolated from bovine retina by established protocols.³² RMECs (passage 5 or earlier) were grown to confluency on 20-mm Petri dishes on native or AGE-FN. To establish the potential for EPCs to heal wounds that are relevant to *in vivo* retinal capillaries a new model was devised. RMEC monolayers were treated with the photodynamic drug verteporfin 0.5 to 1 $\mu\text{g}/\text{mL}$ (Novartis International, Basel, Switzerland). Discrete regions of the monolayer were illuminated by a collimated beam of red light (600 nm)

from a monochromator (Cairn Research, Faversham, UK). The light was fed into the inverted microscope (TE2000; Nikon, Tokyo, Japan) via a fiber optic light guide and a 40 \times objective to create wounds of 1 mm diameter. DiI-labeled EPCs were added to these cultures, and adherent EPCs were counted in six random fields after staining of RMECs with biotinylated BS1-B4 isolectin (Sigma-Aldrich) and streptavidin conjugated AlexaFluor 488 (Invitrogen-Molecular Probes) at the site of the injury after 24 hours of treatment.

EPC Interaction with Resident Endothelium during Wound Repair

RMECs were seeded at a density of 2×10^5 on 0.4- μ m chamber inserts (Transwells; Sigma-Aldrich). Established monolayers were wounded by using verteporfin as described earlier. DiI-labeled EPCs were then added and allowed to interact with both normal and wounded endothelium. EPC-mediated repair of the RMEC monolayer was studied by estimating the transendothelial electric resistance (TEER) after a further 24 hours (Millicell Electric Resistance System; Millipore, Bedford, MA). In addition, the ability of EPCs to integrate with the RMECs and form verifiable tight junctions was studied by immunofluorescent staining with anti-ZO-1 (Zymed-Invitrogen, Paisley, UK) and confocal microscopy.

Statistical Analysis

Data are expressed as the mean \pm SEM. Statistical differences in the mean were assessed with one-way analysis of variance (ANOVA) followed by the Tukey-Kramer post hoc test for multiple comparisons, unless stated otherwise. Univariate analysis of variance was performed to assess the overall effect of RGD treatments on attachment and spreading responses. Chemotaxis data were analyzed by using the Raleigh test for statistical significance. All the statistical analyses were performed with commercial software (SPSS ver. 14.0; SPSS, Chicago, IL, or InStat 3.0; GraphPad Software, San Diego, CA).

RESULTS

Isolation and Characterization of EPC

Isolated EPCs exhibited a typical spindle appearance with distinct colony formations by 48 to 72 hours (Fig. 1A). On phenotypic evaluation, these cells endocytosed acLDL DiI and stained positively with UEA-1 lectin (Fig. 1B) consistent with an endothelial lineage. Flow cytometric analysis revealed that 93.1% of these cells were immunoreactive for VEGFR2 and that 25.4% cells expressed CD34 (Figs. 1D,

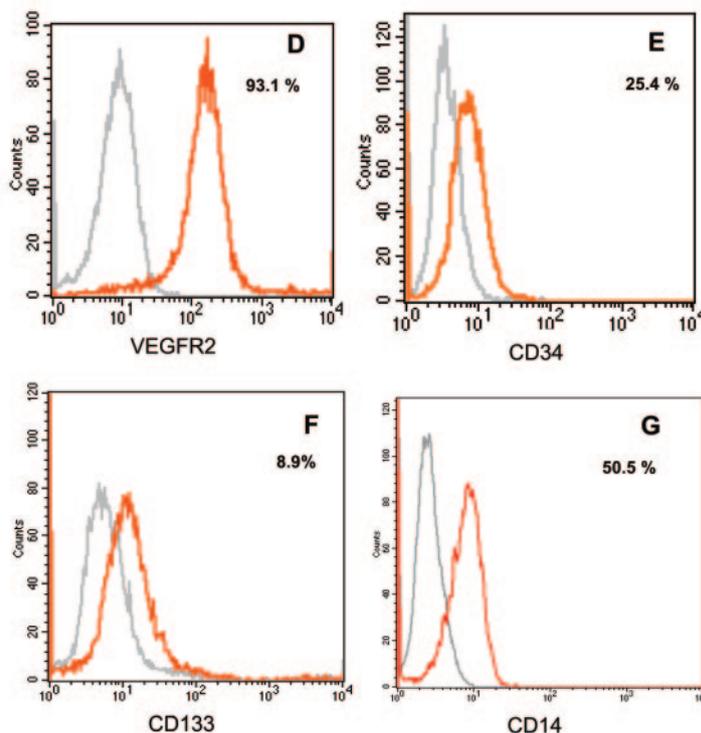
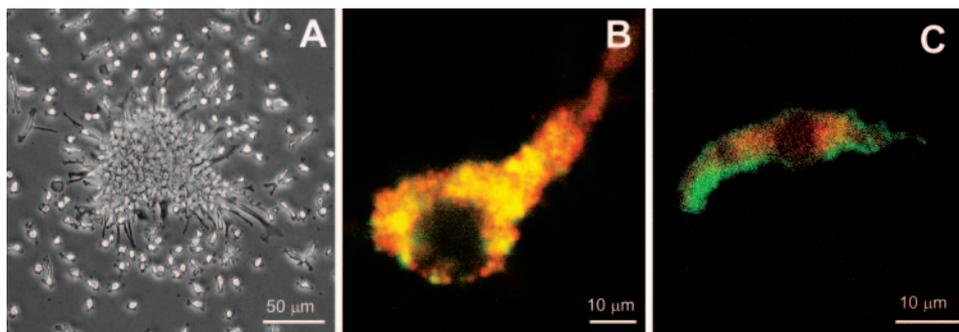


FIGURE 1. Isolation and characterization of EPCs. Colonies of EPCs in culture appeared within 48 to 72 hours of plating (A). A confocal micrograph revealed uptake of acLDL DiI (red) and UEA-1 (green) by EPCs (B), whereas these cells were also readily labeled by DiI (red) and showed CD31 membrane localization (green; C). Flow cytometric analysis of the EPCs was conducted, and the data are presented in histograms; red trace: antibody treatment; gray trace: unlabeled or isotype control (D–G). EPCs show presence of the endothelial lineage marker VEGFR2 (D) and also the hematopoietic stem cell marker CD34 (E). On further analysis 8.9% of the cells were positive for AC133 (F) and more than 50% of cells exhibited monocytic CD14 expression (G).

1E). Also, 50.5% of the adherent population showed positive characteristics for CD14, whereas 8.9% of cells were positive for CD133 (Figs. 1F, 1G).

EPC Dynamics on AGE-Modified Substrate

FN incubated with MGO significantly elevated levels of CEL adducts when compared to nonmodified control substrate (Fig. 2A). HPLC analysis for MGO-derived argpyrimidine showed a retention time of this adduct at 18 minutes. Levels of argpyrimidine increased in proportion to the concentration of MGO (Fig. 2B).

EPC adhesion on AGE-FN was significantly reduced in a concentration-dependent manner with adherent cells at less than 25% of the control value when applied to maximally modified FN. Replenishment of AGE-modified FN with arginine residues in terms of exogenous RGD peptide significantly restored EPC adhesion (Fig. 3A; $P < 0.01$). A similar response

was observed for cell spreading; EPCs on native FN typically exhibited spiked cytoplasmic processes positive for filamentous actin, typical of a normal spreading response. In contrast, cells that had attached to AGE-FN were rounded up in appearance and their actin was not filamentous. Analysis of these cells on the basis of “spread” or “unspread” (Figs. 3Bi, 3Bii) showed a significant reduction in spreading on AGE-FN when compared with the FN-treated control cells ($P < 0.01$). Supplementation of AGE-FN with RGD peptide significantly restored EPC spreading ($P < 0.05$; Fig. 3C).

To explore EPC spreading and substrate interaction further, we quantified phosphorylation of FAK (phospho-FAK). EPCs interacting with FN demonstrated defined points of adhesion that were marked by phosphorylation events to FAK on the basal membrane (Fig. 4A). EPCs introduced to AGE-FN showed significantly less focal adhesion contacts when compared to nonmodified FN control substrate (Fig. 4A). Quantification of

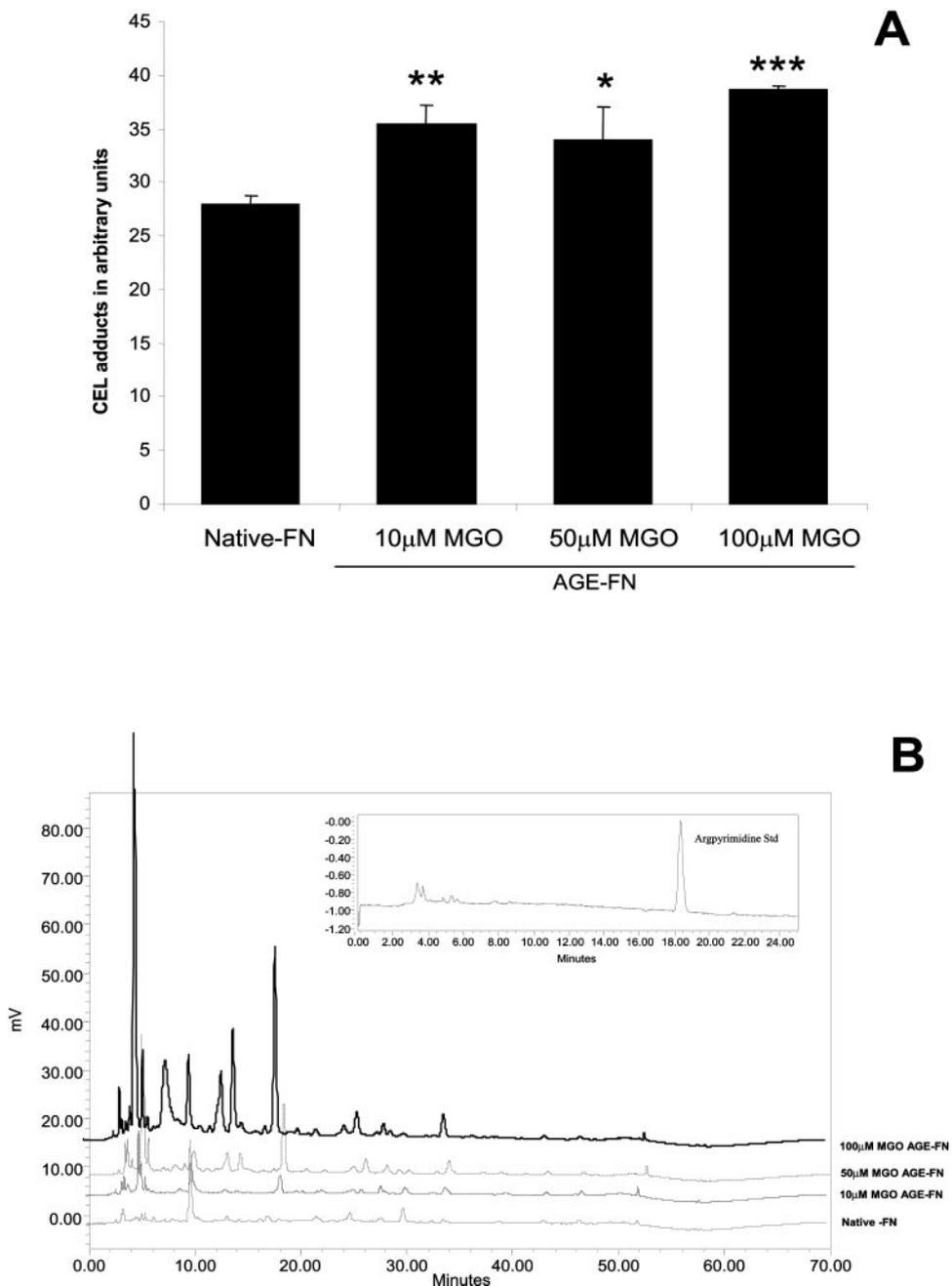


FIGURE 2. Quantification of AGE adducts. Competitive ELISA for CEL adducts showed a concentration-dependent increase in the formation of AGE adducts with the maximum of MGO treatment at 100 µM (A). HPLC analysis of the MGO-derived AGE argpyrimidine was measured and presented as a chromatogram showing the peak for argpyrimidine standard at 18 minutes (*inset*; B). MGO-treated FN exhibited similar retention time but demonstrated a concentration-dependent increase in peak area. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

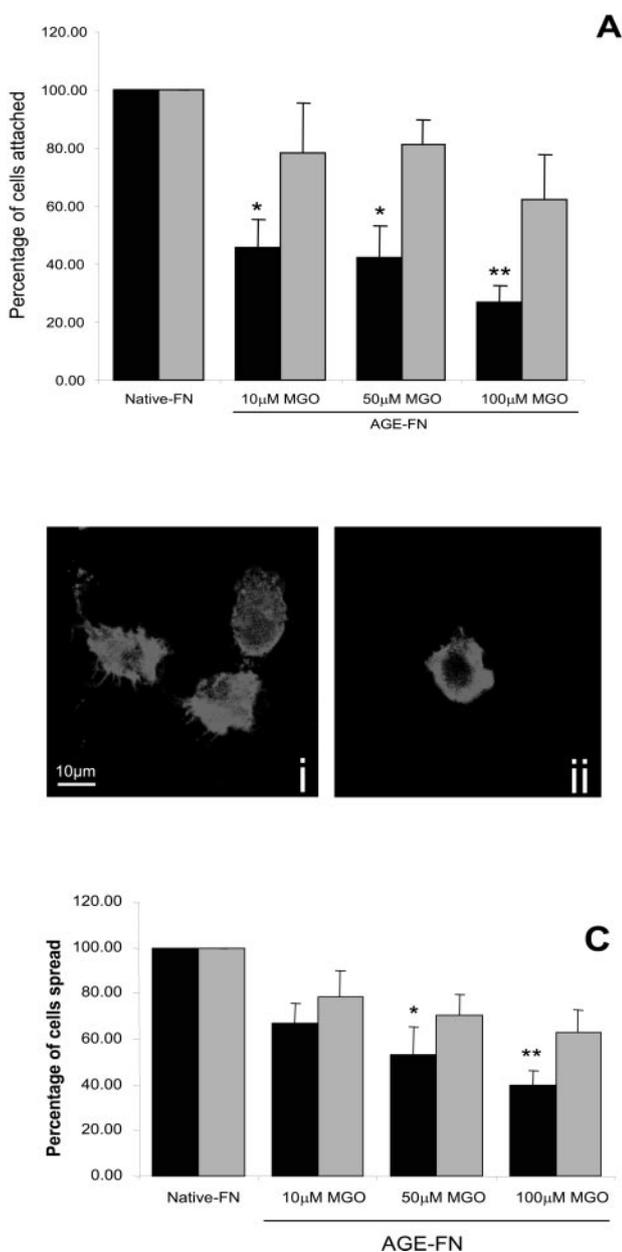


FIGURE 3. EPC dynamics are significantly altered on AGE-FN. EPC attachment is reduced in a concentration-dependent manner with the maximum decrease apparent when FN is most modified by MGO (A). Exogenous RGD treatment of the FN before cell plating significantly augmented attachment (A). EPCs on native, unmodified FN showed defined spreading responses (Bi), whereas cells plated on AGE-FN showed a characteristic rounded appearance (Bii). There was reduced spreading in a concentration-dependent manner when compared to control values, whereas supplementation of AGE-FN with RGD peptide restored this EPC response (C).

these contact points showed 3.15 ± 1.1 phospho-FAK⁺ focal adhesions on native FN per cell, which significantly dropped to 2.3 ± 0.98 and 1.96 ± 0.92 per cell in the 10- and 50- μ M MGO-treated AGE-FN groups, respectively (Fig. 4B). Exogenous RGD significantly restored phospho-FAK⁺ focal adhesions to 3.55 ± 2.04 and 3.6 ± 1.66 ($P < 0.001$) on AGE-FN-treated cells (Fig. 4B).

EPC Chemotaxis

To investigate the ability of EPCs to migrate in response to a known chemotactic agent for these cells, we performed the

Dunn chemotaxis assay. When there was no SDF-1 gradient, EPCs on native FN moved randomly (Fig. 5Ai). In the presence of SDF-1, EPC migration showed a trajectory that followed the concentration gradient ($P < 0.05$; Fig. 5Aii). This chemotactic response was abolished on AGE-FN (Fig. 5Aiii) and was significantly restored by prior treatment of the AGE-FN with RGD peptide (Fig. 5Aiv). The speed of EPC migration is a function of mean distance traversed by individual cells (micrometers over 10 minutes), which on the increasingly AGE-modified substrates paralleled the chemotaxis findings. There was a marked slowing of EPC chemotaxis on AGE-FN, a response that was significantly reversed by RGD supplementation (Fig. 5B; $P < 0.001$).

In Vitro Model for Retinal Endothelial Cell Injury

Confluent RMEC monolayers treated with verteporfin (in non-illuminated conditions) showed no obvious cytotoxic response. However, the focusing of a red light source on a defined region of the monolayer caused an activation of verteporfin, culminating in highly circumscribed apoptotic responses by the light-exposed RMECs. The advantage of such wounds in comparison to those produced by scraping is that they have a reproducible area and the underlying cell-associated matrix remains undisturbed. Apoptotic cells showed a characteristic rounded-up, phase-bright configuration and

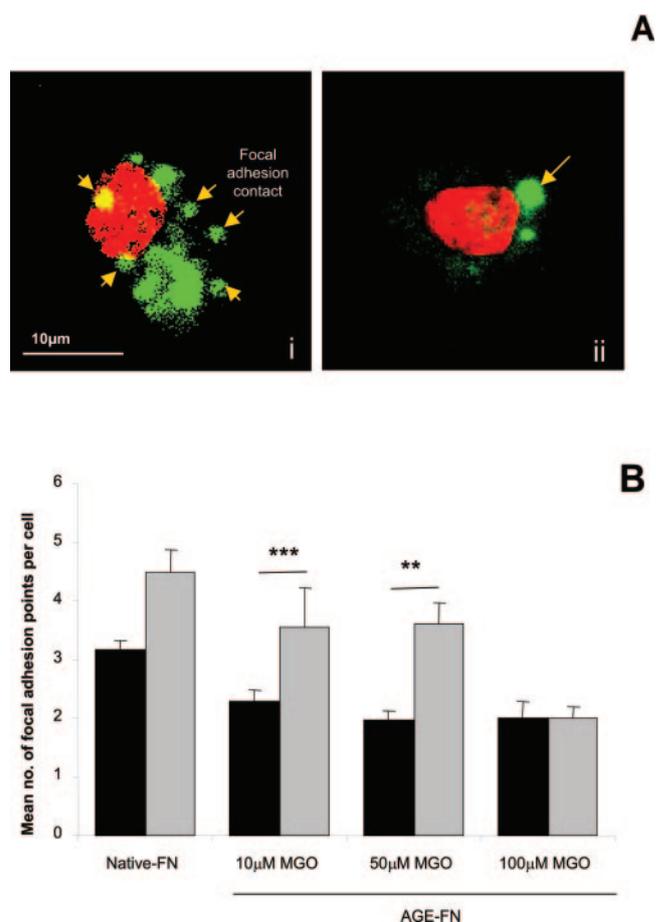


FIGURE 4. Focal adhesion points on EPCs. Development of focal adhesion contacts after attachment of EPCs on native FN (Ai, arrows). This number was reduced on AGE-modified RN (Aii). Phosphorylated FAK contacts decreased on AGE-FN when compared with native-FN (B), whereas supplementation with RGD peptide improved this signaling event on the basal aspect of the spread EPCs. ** $P < 0.01$, *** $P < 0.001$ (■, RGD⁻; □, RGD⁺).

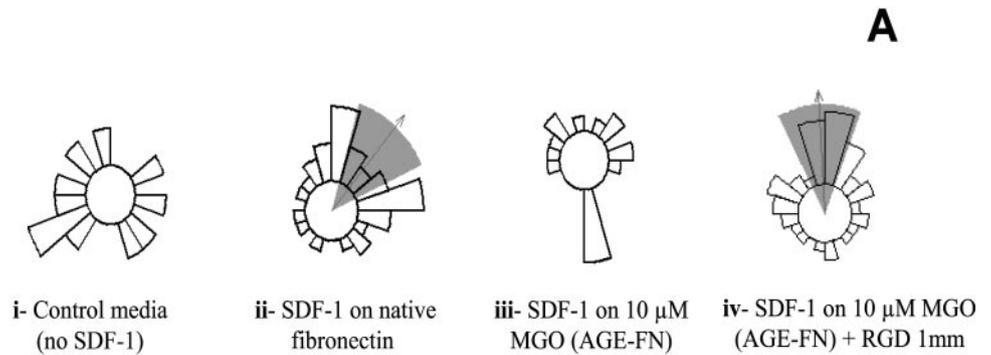
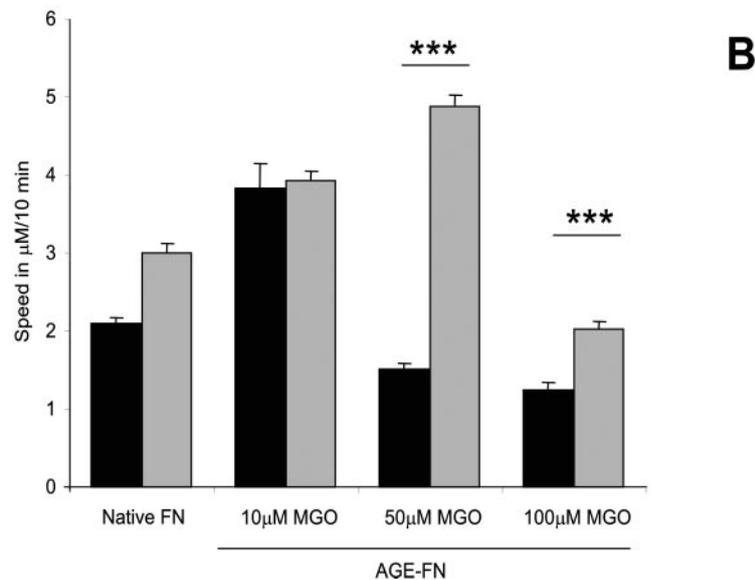


FIGURE 5. EPC migration along an SDF-1 gradient was impaired on AGE-FN. Chemotaxis was studied by using the Dunn chamber assay and the data are represented by circular histograms showing cell displacements for EPC chemotaxis. The histograms show the proportion of cells migrating within 18° intervals with the source of chemoattractant at the top of the histogram (A). In the absence of SDF-1, EPCs on native FN showed no specific directional migration (Ai). The presence of an SDF-1 gradient modulated a specific directional migration of EPCs, as shown by the *arrow* and *gray* segment, indicating the mean significant migration direction and its 99% CI (Aii). EPCs on AGE-FN (10 μ M MGO) did not show any chemoattraction to SDF-1 (Aiii), whereas the presence of RGD peptide restored the migratory response on the AGE-FN to levels observed on native FN (Aiv). The Dunn chamber assay allows speed of migration to be assessed, and the speed was significantly reduced on AGE-FN, whereas supplementation of substrates with RGD peptides restored normal speed of migration. *** $P < 0.001$ (■, RGD⁻; □, RGD⁺).



eventually lifted from the substrate (Fig. 6A). These cells also showed fragmented nuclei on PI labeling (data not shown).

Addition of DiI-labeled EPCs to wounded RMEC monolayers showed specific targeting at the site of injury as early as 2 hours after they were added to the cultures. Quantitative analysis showed that there were fivefold more EPCs in the denuded, substrate-exposed areas than in the regions of intact endothelial monolayer (Figs. 6B, 6C; $P < 0.001$). BS1-B4 isolectin staining 24 to 48 hours after addition of EPCs to wounded RMEC monolayers revealed that EPCs integrated with the resident RMECs at the site of injury and contributed to the overall healing response, although the number of cells incorporated was disproportionately fewer than the initial population of EPCs attached at the wound site (Fig. 6C). RMEC monolayers grown on AGE-FN reached confluence, but more slowly than when grown on native-FN. EPC homing to denuded areas was markedly reduced when compared with control cells ($P < 0.05$). This response was dependent on the degree of AGE modification and EPCs applied to wounded monolayers grown on maximally modified 100 μ M MGO-treated FN showed a more than 75% reduction in EPC homing compared with wounds overlying control FN substrate ($P < 0.05$; Fig. 7).

EPC Restoration of Endothelial Barrier Properties

There was no significant difference in TEER recordings between confluent RMEC monolayer grown on AGE-FN (50 μ M MGO) and native-FN (40.68 ± 16.09 vs. 45.56 ± 6.31 Ω/cm^2). After verteporfin wounding and successive repair by EPCs, RMECs propagated on AGE-FN restored endothelial barrier with observed TEER as 34.3 ± 3.24 Ω/cm^2 , which was dropped to 16.8 ± 2.8 Ω/cm^2 in 50 μ M MGO-treated AGE-FN (Fig. 8A). Addition of exogenous RGD peptide to the substrate substantially restored TEER to 45.1 ± 6.7 Ω/cm^2 (Fig. 7A; $P < 0.05$). The contribution of EPCs to this microvascular barrier was also assessed by using ZO-1 immunolocalization as an indicator of tight junction integrity. RMECs showed typical junctional staining (Fig. 8Bi). Several DiI-labeled EPCs appeared to integrate into the RMEC monolayer and formed tight junctions with the resident cells, as indicated by the cytoplasmic localization of ZO-1 (Fig. 8Biii). However, it was also apparent that many EPCs did not differentiate but rather were localized in the space beneath the RMEC monolayer (Fig. 8Bii).

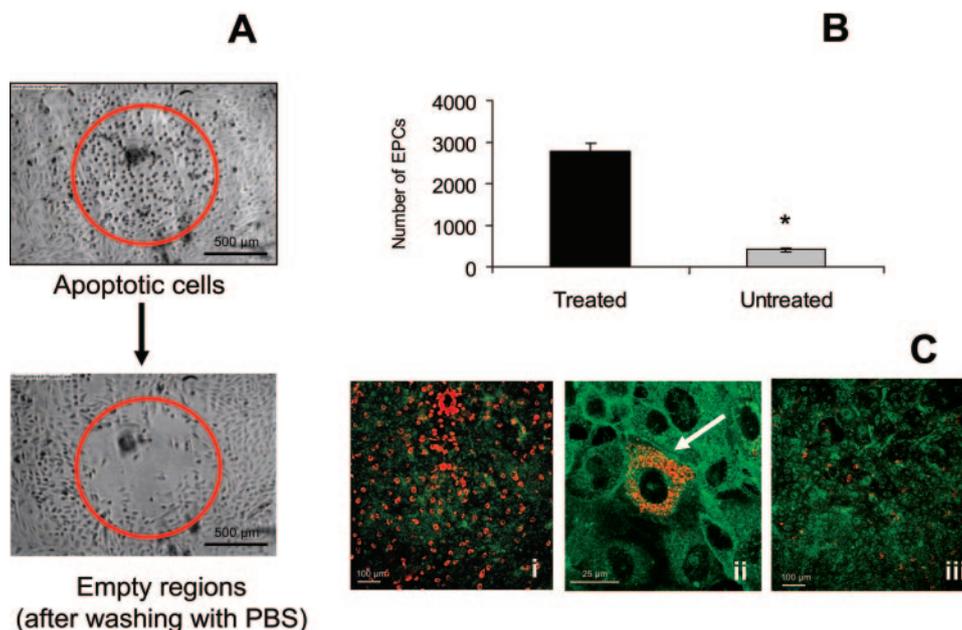


FIGURE 6. Verteporfin model for the study of endothelial cell apoptosis and repair by EPCs. The photodynamic drug verteporfin was readily endocytosed by RMECs; and, when subsequently exposed to a single beam of red light, there was a coordinated apoptotic response in exposed cells. When the beam was focused on a discrete region (A), the apoptosis is also discrete (*red circled region*), whereas other areas of the monolayer remain intact (A). When these RMEC monolayers are washed with PBS, the denuded endothelium and exposed substrate were apparent (A). When EPCs were added to this model system, fivefold more DiI-labeled cells were observed at the wound site than in the distant (unharmed) regions on the same plate (B). Staining cocultures with BS1-B4 isolectin (*green*) showed that EPCs (*red*) homed to wound sites (Ci) and participated in wound healing by forming a part of the monolayer (Cii, *arrow*). Distant regions on the same plate showed comparatively few EPCs (Ciii).

DISCUSSION

EPCs make a significant contribution to microvascular repair, although as diabetes progresses, the normal function of these cells is significantly impaired.^{33,34} EPC dysfunction in diabetes may be linked to abnormalities in the resident vessels into which EPCs incorporate, although it may also reside in an inherent diabetes-mediated defect in the progenitor cell at

various points in the differentiation pathway.²⁶ While acknowledging the important contribution of an inherent pathophysiology in diabetic EPCs, the present study has investigated the relative contribution of diabetes-linked AGE-modifications on vascular BM and how this may impair recruitment and incorporation to sites of endothelial loss. Based on the promising results of the REPAIR-AMI (Reinfusion of Enriched Progenitor Cells and Infarct Remodeling in Acute Myocardial Infarction) trial,³⁵ introduction of nondiabetic EPCs into diabetic patients is being considered to promote vascular repair and reperfusion of the ischemic retina. Our study further explored the feasibility of such a therapeutic option in diabetic patients exhibiting extensive protein cross-linking of BM due to AGE accumulation. With the use of DR as a model for studying ischemic repair by EPCs, AGE adducts on the retinal microvascular BM significantly impaired EPC-mediated reparative function in vitro.

AGE accumulation on the BM of retinal capillaries during diabetes is well recognized³⁶ and can influence vascular cell survival.¹⁰ Modification of proteins by advanced glycation constitutes a key pathogenic event in DR.³⁷ Accumulation of these adducts can be four times more with FN than with collagen.³⁸ MGO is a significant precursor for AGE formation, which induces rapid modification of arginine residues in many proteins of the diabetic retina.³⁹ MGO is elevated in diabetic serum,⁴⁰ and we have recently shown that MGO-derived AGEs can form rapidly on the retinal vessels of diabetic mice.²⁹ MGO modification of FN is probably significant, as it is involved in attachment and spreading of adherent cells on vascular BM.⁴¹ Moreover, FN provides key cell-recognition motifs such as RGD or GRGDSP that activate integrin $\alpha 5 \beta 1$ signaling and hence EPC differentiation compared with collagen or vitronectin.¹⁵ The RGD motif is important for cell recognition and pro-survival

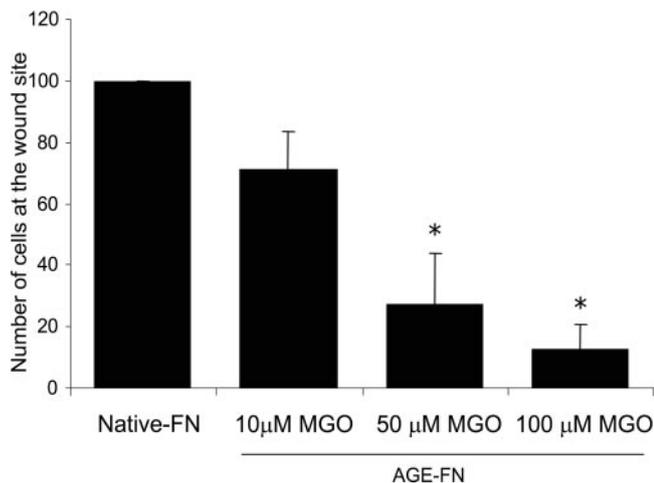


FIGURE 7. EPC attachment to denuded RMEC monolayers was attenuated by the presence of an AGE-modified substrate. EPCs added to verteporfin-wounded RMEC monolayers showed reduced attachment to the wound site when the FN substrate was AGE modified. Attachment to AGE-FN (modification by 100 μ M MGO) was only 15% of that observed with the native RN ($P < 0.05$).

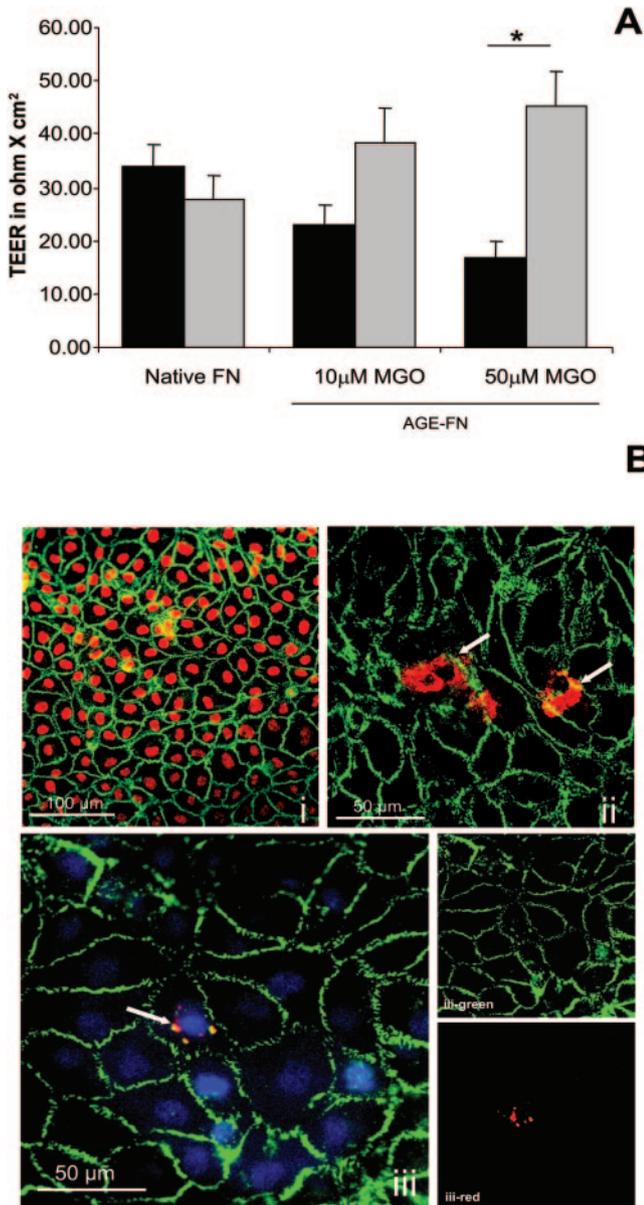


FIGURE 8. EPCs play a dual role in promoting endothelial monolayer integrity. As a functional assessment of EPC reparative function on wounded RMEC monolayers, the TEER assay was performed as an assessment of barrier properties. When RMECs were grown on AGE-FN, the barrier function was significantly reduced when compared with native FN (A). This AGE-mediated impairment was significantly reduced when the denuded areas are treated with exogenous RGD peptide (A). * $P < 0.05$, (■ RGD⁻; □ RGD⁺). In wounded monolayers without the presence of EPCs, the retinal endothelial cells formed a monolayer with tight junction integrity, as depicted by ZO-1 immunolocalization at the cell junctions (green; nuclei stained with propidium iodide (Bi, red). When added to the denuded RMECs, many EPCs did not incorporate with the resident endothelium but appear located in the basal aspect of the monolayer (Bii, arrows). These cells did not form tight junctions (Bii). Other EPCs incorporated directly into the RMEC monolayer as indicated by cytoplasmic localization of DiI (red; arrow) and ZO-1 labeling at the junctions (green; DAPI-stained nuclei are shown in blue in the merged micrograph (Biii).

signaling.⁴² Furthermore, the arginine residue of RGD is highly susceptible to MGO-derived AGE adduct formation, and these modifications reduce adhesion and spreading activity of adherent cells.^{11,43} It should be appreciated that FN is not the only component of vascular BM, and it is likely that AGE-modifica-

tion of other proteins, especially at their arginine and lysine residues, could also have a profound influence on resident capillary function and also EPC reparative capacity.

Diabetes-related modification of the vascular BM results in impaired endothelial function⁴⁴ and differentiation.⁴⁵ Building on these findings, and in an attempt to investigate the complex interplay between capillary endothelium, substrate, and reparative EPC function, we have developed a novel in vitro model whereby endothelial cell apoptosis can be induced while leaving large segments of the monolayer intact. This approach mimics disease known to occur in retinal capillaries as diabetes progresses, where endothelial cells undergo apoptosis in response to a range of biochemical insults.^{21,46} Although the model provides a unique insight into how EPCs interact with exposed BM and resident RMECs, it is unable to provide dynamics of EPC interactions with RMEC due to limitations of coculture. In ongoing research in our laboratory, we are investigating important mechanisms underpinning EPC homing and differentiation in this system. In the present study, this system shows the detrimental impact of substrate AGEs on EPC incorporation when gaps appear in the monolayer.

EPCs derived from diabetic sources are unable to revascularize ischemic retina¹⁸ via vascular repair or physiological intraretinal angiogenesis. Our ongoing experiments are exploring the possibility of BM modification as a component mechanism behind this deficit. Preliminary data from diabetic patients show that diabetic EPCs lack functional ability and are unable to form endothelial tubes on AGE-modified BM. When explored further, this deficit is shown to be attributable to decreased expression of FAK, upregulation of RAGE and/or impaired nitric oxide activity. Future experiments with multiple diabetic donors will provide a clear-cut understanding of the exact nature of diabetic CD34⁺ cells in diabetic retinopathy.

At present, the characteristics of circulating EPCs are not completely known, and the nature and origin of endothelial progenitor cells is controversial. Many studies concentrate on the CD34⁺ population, but CD34⁻ cells (possibly with the CD14⁺ myeloid subset which is resilient during diabetes⁴⁷), also induce vascular repair in diabetic ischemic wounds. Hyperglycemia may compromise the vasoreparative function of CD34⁺ cells, and in such cases freshly isolated CD14⁺ cells may serve as an alternative therapeutic option for the repair of diabetic vessels.⁴⁸ However, there may be a need to treat simultaneously with CD34⁺ populations to achieve a positive therapeutic outcome with CD14⁺ myeloid cells, perhaps due to improved leukocyte-leukocyte interaction.⁴⁹ Culturing conditions may combine all EPC subsets directing them toward endothelial differentiation,⁵⁰ possibly due to costimulation of at least one subset of the EPC population. It is likely that our EPCs are a mixed population of progenitors that show differing proliferative potential and tube-forming properties.⁵¹ Some cells appear to differentiate actively and directly contribute to endothelialization of wound sites, whereas some EPCs did not participate directly and were located underneath the monolayer. However, the overall response was best reflected in terms of restoration of endothelial cell integrity. It is suggested that these cells may stimulate endothelial repair through the expression of angiogenic growth factors and chemokines⁵² or may possess a role of stabilizing retinal neovasculature.⁵³ Future studies on the role of EPCs in diabetic microvasculopathy should take into account the distinct clonal nature and diverse properties of cells that have been traditionally termed endothelial progenitors.

Differentiation and incorporation of EPCs into vessels is controlled by complex basement membrane-associated interactions¹⁵ that include $\alpha v \beta 3$ and $\alpha v \beta 5$ signaling and activation of upstream effectors such as FAK.⁵⁴ It appears that the impaired EPC adhesion, FAK phosphorylation events, and chemo-

tactic responses to SDF-1 in the present study are related to reduced cellular recognition of this motif by cells. This assertion is explained by the concept that AGE-modification of the RGD domain in FN interferes with signaling via the integrin receptor $\alpha 5\beta 1$.⁵⁵ The ability of RGD peptides to reverse many of the dysfunctional matrix-related responses of EPCs and promote vasoreparative function strengthens this concept and suggests that these exogenous peptides replenish critical cell-recognition motifs that are AGE modified (damaged).

In conclusion, AGE modification of BM components has an inhibitory effect on EPC attachment, spreading, and migration. This effect may be linked to the modification of key RGD cell-attachment domains in vascular cell substrates at the site of vascular injury. Our results suggest that AGE modification of FN may have a role in the pathogenesis of diabetic retinopathy.

Acknowledgments

The authors thank Gary Coleman and Maria O'Rourke for technical help and Maurice McClelland and his staff at the Northern Ireland Blood Transfusion Services for supplying blood samples.

References

- Roy S, Sala R, Cagliero E, Lorenzi M. Overexpression of fibronectin induced by diabetes or high glucose: phenomenon with a memory. *Proc Natl Acad Sci USA*. 1990;87:404-408.
- Chen S, Mukherjee S, Chakraborty C, Chakrabarti S. High glucose-induced, endothelin-dependent fibronectin synthesis is mediated via NF-kappa B and AP-1. *Am J Physiol*. 2003;284:C263-C272.
- Roy S, Maiello M, Lorenzi M. Increased expression of basement membrane collagen in human diabetic retinopathy. *J Clin Invest*. 1994;93:438-442.
- Cagliero E, Roth T, Roy S, Lorenzi M. Characteristics and mechanisms of high-glucose-induced overexpression of basement membrane components in cultured human endothelial cells. *Diabetes*. 1991;40:102-110.
- Roy S, Cagliero E, Lorenzi M. Fibronectin overexpression in retinal microvessels of patients with diabetes. *Invest Ophthalmol Vis Sci*. 1996;37:258-266.
- Gardiner TA, Anderson HR, Stitt AW. Inhibition of advanced glycation end-products protects against retinal capillary basement membrane expansion during long-term diabetes. *J Pathol*. 2003;201:328-333.
- Stitt AW, Li YM, Gardiner TA, Bucala R, Archer DB, Vlassara H. Advanced glycation end products (AGEs) co-localize with AGE receptors in the retinal vasculature of diabetic and of AGE-infused rats. *Am J Pathol*. 1997;150:523-531.
- Hammes HP, Weiss A, Hess S, et al. Modification of vitronectin by advanced glycation alters functional properties in vitro and in the diabetic retina. *Lab Invest*. 1996;75:325-338.
- McKenna DJ, Nelson J, Stitt AW. Advanced glycation alters expression of the 67kDa laminin receptor in retinal microvascular endothelial cells. *Life Sci*. 2001;68:2695-2703.
- Stitt AW, Hughes SJ, Canning P, et al. Substrates modified by advanced glycation end-products cause dysfunction and death in retinal pericytes by reducing survival signals mediated by platelet-derived growth factor. *Diabetologia*. 2004;47:1735-1746.
- Liu B, Bhat M, Padival AK, Smith DG, Nagaraj RH. Effect of dicarbonyl modification of fibronectin on retinal capillary pericytes. *Invest Ophthalmol Vis Sci*. 2004;45:1983-1995.
- Dobler D, Ahmed N, Song L, Eboigbodin KE, Thornalley PJ. Increased dicarbonyl metabolism in endothelial cells in hyperglycemia induces anoikis and impairs angiogenesis by RGD and GFOGER motif modification. *Diabetes*. 2006;55:1961-1969.
- Asahara T, Murohara T, Sullivan A, et al. Isolation of putative progenitor endothelial cells for angiogenesis. *Science*. 1997;275:964-967.
- Rafii S, Lyden D. Therapeutic stem and progenitor cell transplantation for organ vascularization and regeneration. *Nat Med*. 2003;9:702-712.
- Wijelath ES, Rahman S, Murray J, Patel Y, Savidge G, Sobel M. Fibronectin promotes VEGF-induced CD34 cell differentiation into endothelial cells. *J Vasc Surg*. 2004;39:655-660.
- Wijelath ES, Murray J, Rahman S, et al. Novel vascular endothelial growth factor binding domains of fibronectin enhance vascular endothelial growth factor biological activity. *Circ Res*. 2002;91:25-31.
- Dorrell MI, Otani A, Aguilar E, Moreno SK, Friedlander M. Adult bone marrow-derived stem cells use R-cadherin to target sites of neovascularization in the developing retina. *Blood*. 2004;103:3420-3427.
- Caballero S, Sengupta N, Afzal A, et al. Ischemic vascular damage can be repaired by healthy, but not diabetic, endothelial progenitor cells. *Diabetes*. 2007;56:960-967.
- Grant MB, May WS, Caballero S, et al. Adult hematopoietic stem cells provide functional hemangioblast activity during retinal neovascularization. *Nat Med*. 2002;8:607-612.
- Otani A, Kinder K, Ewalt K, Otero FJ, Schimmel P, Friedlander M. Bone marrow-derived stem cells target retinal astrocytes and can promote or inhibit retinal angiogenesis. *Nat Med*. 2002;8:1004-1010.
- Mizutani M, Kern TS, Lorenzi M. Accelerated death of retinal microvascular cells in human and experimental diabetic retinopathy. *J Clin Invest*. 1996;97:2883-2890.
- Sharma NK, Gardiner TA, Archer DB. A morphologic and autoradiographic study of cell death and regeneration in the retinal microvasculature of normal and diabetic rats. *Am J Ophthalmol*. 1985;100:51-60.
- Rubin H. The disparity between human cell senescence in vitro and lifelong replication in vivo. *Nat Biotechnol*. 2002;20:675-681.
- Schachinger V, Erbs S, Elsasser A, et al. Intracoronary bone marrow-derived progenitor cells in acute myocardial infarction. *N Engl J Med*. 2006;355:1210-1221.
- Chen YH, Lin SJ, Lin FY, et al. High glucose impairs early and late endothelial progenitor cells by modifying nitric oxide-related but not oxidative stress-mediated mechanisms. *Diabetes*. 2007;56:1559-1568.
- Segal MS, Shah R, Afzal A, et al. Nitric oxide cytoskeletal-induced alterations reverse the endothelial progenitor cell migratory defect associated with diabetes. *Diabetes*. 2006;55:102-109.
- Kalka C, Masuda H, Takahashi T, et al. Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. *Proc Natl Acad Sci USA*. 2000;97:3422-3427.
- Degenhardt TP, Alderson NL, Arrington DD, et al. Pyridoxamine inhibits early renal disease and dyslipidemia in the streptozotocin-diabetic rat. *Kidney Int*. 2002;61:939-950.
- Canning P, Glenn JV, Hsu DK, Liu FT, Gardiner TA, Stitt AW. Inhibition of advanced glycation and absence of galectin-3 prevent blood-retinal barrier dysfunction during short-term diabetes. *Exp Diabetes Res*. 2007;2007:51837.
- Krishnamurti U, Rondeau E, Sraer JD, Michael AF, Tsilibary EC. Alterations in human glomerular epithelial cells interacting with nonenzymatically glycosylated matrix. *J Biol Chem*. 1997;272:27966-27970.
- Li G, Cui Y, McIlmurray L, Allen WE, Wang H. rhBMP-2, rhVEGF(165), rhPTN and thrombin-related peptide, TP508 induce chemotaxis of human osteoblasts and microvascular endothelial cells. *J Orthop Res*. 2005;23:680-685.
- Stitt AW, Chakravarthy U, Archer DB, Gardiner TA. Increased endocytosis in retinal vascular endothelial cells grown in high glucose medium is modulated by inhibitors of nonenzymatic glycosylation. *Diabetologia*. 1995;38:1271-1275.
- Loomans CJ, de Koning EJ, Staal FJ, et al. Endothelial progenitor cell dysfunction: a novel concept in the pathogenesis of vascular complications of type 1 diabetes. *Diabetes*. 2004;53:195-199.
- Tamarat R, Silvestre JS, Le Ricousse-Roussanne S, et al. Impairment in ischemia-induced neovascularization in diabetes: bone marrow mononuclear cell dysfunction and therapeutic potential of placenta growth factor treatment. *Am J Pathol*. 2004;164:457-466.
- Schachinger V, Erbs S, Elsasser A, et al. Improved clinical outcome after intracoronary administration of bone-marrow-derived progenitor cells in acute myocardial infarction: final 1-year results of the REPAIR-AMI trial. *Eur Heart J*. 2006;27:2775-2783.

36. Gardiner TA, Stitt AW, Anderson HR, Archer DB. Selective loss of vascular smooth muscle cells in the retinal microcirculation of diabetic dogs. *Br J Ophthalmol*. 1994;78:54-60.
37. Stitt AW. The maillard reaction in eye diseases. *Ann NY Acad Sci*. 2005;1043:582-597.
38. Tarsio JF, Reger LA, Furcht LT. Decreased interaction of fibronectin, type IV collagen, and heparin due to nonenzymatic glycation: implications for diabetes mellitus. *Biochemistry*. 1987;26:1014-1020.
39. Padayatti PS, Jiang C, Glomb MA, Uchida K, Nagaraj RH. High concentrations of glucose induce synthesis of argpyrimidine in retinal endothelial cells. *Curr Eye Res*. 2001;23:106-115.
40. Fosmark DS, Torjesen PA, Kilhovd BK, et al. Increased serum levels of the specific advanced glycation end product methylglyoxal-derived hydroimidazolone are associated with retinopathy in patients with type 2 diabetes mellitus. *Metabolism*. 2006;55:232-236.
41. Hayden MR, Sowers JR, Tyagi SC. The central role of vascular extracellular matrix and basement membrane remodeling in metabolic syndrome and type 2 diabetes: the matrix preloaded. *Cardiovasc Diabetol*. 2005;4:9.
42. Pierschbacher MD, Ruoslahti E. Variants of the cell recognition site of fibronectin that retain attachment-promoting activity. *Proc Natl Acad Sci USA*. 1984;81:5985-5988.
43. Sakata N, Sasatomi Y, Meng J, et al. Possible involvement of altered RGD sequence in reduced adhesive and spreading activities of advanced glycation end product-modified fibronectin to vascular smooth muscle cells. *Connect Tissue Res*. 2000;41:213-228.
44. Bobbink IW, de Boer HC, Tekelenburg WL, Banga JD, de Groot PG. Effect of extracellular matrix glycation on endothelial cell adhesion and spreading: involvement of vitronectin. *Diabetes*. 1997;46:87-93.
45. Kuzuya M, Satake S, Miura H, Hayashi T, Iguchi A. Inhibition of endothelial cell differentiation on a glycosylated reconstituted basement membrane complex. *Exp Cell Res*. 1996;226:336-345.
46. Zheng L, Szabo C, Kern TS. Poly(ADP-ribose) polymerase is involved in the development of diabetic retinopathy via regulation of nuclear factor-kappaB. *Diabetes*. 2004;53:2960-2967.
47. Awad O, Jiao C, Ma N, Dunnwald M, Schatteman GC. Obese diabetic mouse environment differentially affects primitive and monocytic endothelial cell progenitors. *Stem Cells*. 2005;23:575-583.
48. Awad O, Dedkov EI, Jiao C, Bloomer S, Tomanek RJ, Schatteman GC. Differential healing activities of CD34+ and CD14+ endothelial cell progenitors. *Arterioscler Thromb Vasc Biol*. 2006;26:758-764.
49. Harraz M, Jiao C, Hanlon HD, Hartley RS, Schatteman GC. CD34⁻ blood-derived human endothelial cell progenitors. *Stem Cells*. 2001;19:304-312.
50. Urbich C, Heeschen C, Aicher A, Dernbach E, Zeiher AM, Dimmeler S. Relevance of monocytic features for neovascularization capacity of circulating endothelial progenitor cells. *Circulation*. 2003;108:2511-2516.
51. Yoder MC, Mead LE, Prater D, et al. Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals. *Blood*. 2007;109:1801-1809.
52. Hur J, Yoon CH, Kim HS, et al. Characterization of two types of endothelial progenitor cells and their different contributions to neovascularogenesis. *Arterioscler Thromb Vasc Biol*. 2004;24:288-293.
53. Ritter MR, Banin E, Moreno SK, Aguilar E, Dorrell MI, Friedlander M. Myeloid progenitors differentiate into microglia and promote vascular repair in a model of ischemic retinopathy. *J Clin Invest*. 2006;116:3266-3276.
54. Walter DH, Rittig K, Bahlmann FH, et al. Statin therapy accelerates reendothelialization: a novel effect involving mobilization and incorporation of bone marrow-derived endothelial progenitor cells. *Circulation*. 2002;105:3017-3024.
55. Schwarzbauer JE, Sechler JL. Fibronectin fibrillogenesis: a paradigm for extracellular matrix assembly. *Curr Opin Cell Biol*. 1999;11:622-627.