Impaired Retinal Angiogenesis in Diabetes

Role of Advanced Glycation End Products and Galectin-3

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Suppression of angiogenesis during diabetes is a recognized phenomenon but is less appreciated within the context of diabetic retinopathy. The current study has investigated regulation of retinal angiogenesis by diabetic serum and determined if advanced glycation end products (AGEs) could modulate this response, possibly via AGE-receptor interactions. A novel in vitro model of retinal angiogenesis was developed and the ability of diabetic sera to regulate this process was quantified. AGE-modified serum albumin was prepared according to a range of protocols, and these were also analyzed along with neutralization of the AGE receptors galectin-3 and RAGE. Retinal ischemia and neovascularization were also studied in a murine model of oxygen-induced proliferative retinopathy (OIR) in wild-type and galectin-3 knockout mice (gal3$^{-/-}$) after perfusion of preformed AGEs. Serum from nondiabetic patients showed significantly more angiogenic potential than diabetic serum ($P < 0.0001$) and within the diabetic group, poor glycemic control resulted in more AGEs but less angiogenic potential than tight control ($P < 0.01$). AGE-modified albumin caused a dose-dependent inhibition of angiogenesis ($P < 0.001$), and AGE receptor neutralization significantly reversed the AGE-mediated suppression of angiogenesis ($P < 0.01$). AGE-treated wild-type mice showed a significant increase in inner retinal ischemia and a reduction in neovascularization compared with non-AGE controls ($P < 0.001$). However, ablation of galectin-3 abolished the AGE-mediated increase in retinal ischemia and restored the neovascular response to that seen in controls. The data suggest a significant suppression of angiogenesis by the retinal microvasculature during diabetes and implicate AGEs and AGE-receptor interactions in its causation. *Diabetes* 53: 785–794, 2005

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AGE, advanced glycation end product; CEL, carboxyethyl-lysine; CML, carboxymethyl-lysine; DMEM, Dulbecco's modified Eagle's medium; GA, glycoaldehyde; MSA, mouse serum albumin; RAGE, receptor for AGEs; RMEC, retinal microvascular endothelial cell; VEGF, vascular endothelial growth factor.

Vascular complications remain the foremost cause of morbidity and mortality during diabetes (1). Progressive vasodegeneration in microvascular beds is the major underlying factor in initiation and progression of diabetic nephropathy, male impotence, neuropathy, and retinopathy (1,2). In parallel with vasodegenerative change, the phenomenon of impaired new vessel growth in the diabetic state is well recognized, whether induced by local ischemia and/or inflammation. As a result, diabetic patients suffer depressed wound healing and granulation responses, as well as exacerbated peripheral limb ischemia and cardiac mortality through reduced collateral development (3,4). Although preretinal neovascularization is a major complication of diabetic retinopathy, this only arises after considerable capillary drop-out and widespread inner retinal ischemia, which eventually drives a potent growth factor–modulated angiogenic response (5). Indeed, it is apparent that a chronic deficiency in intraretinal new-vessel formation in the face of increasing ischemia makes a major contribution toward progression to the sight-threatening proliferative stages of diabetic retinopathy.

The pathophysiological mechanisms underlying impaired angiogenic potential during diabetes remain largely unelucidated. Among the proposed hyperglycemia-mediated mechanisms, there is accumulating evidence that enhanced formation of advanced glycation end products (AGEs) and activation of AGE receptors in the diabetic state may contribute to impaired angiogenic potential. It has been demonstrated that AGEs attenuate angiogenic processes in vitro (6,7), while in vivo inhibition of AGE formation in diabetic mice can restore ischemia-induced angiogenesis in peripheral limbs (8). Furthermore, neutralization of the receptor for AGEs (RAGE) can restore angiogenic potential during wound healing in diabetic mice (9). There is also some evidence that AGE modification of vasogenic growth factors, within the context of hyperglycemia, impairs their angiogenic potential both in vitro (10,11) and in vivo (12). However, the angiogenic role of AGEs remains somewhat controversial, with several studies reporting that these adducts can promote aspects of the angiogenic process in vitro, including stimulation of endothelial cell proliferation (13) and tube formation (14,15), perhaps though the induction of the angiogenic peptide vascular endothelial growth factor (VEGF) (15).

While much is understood about the mechanisms underlying the vasodegenerative and vasoproliferative etiology

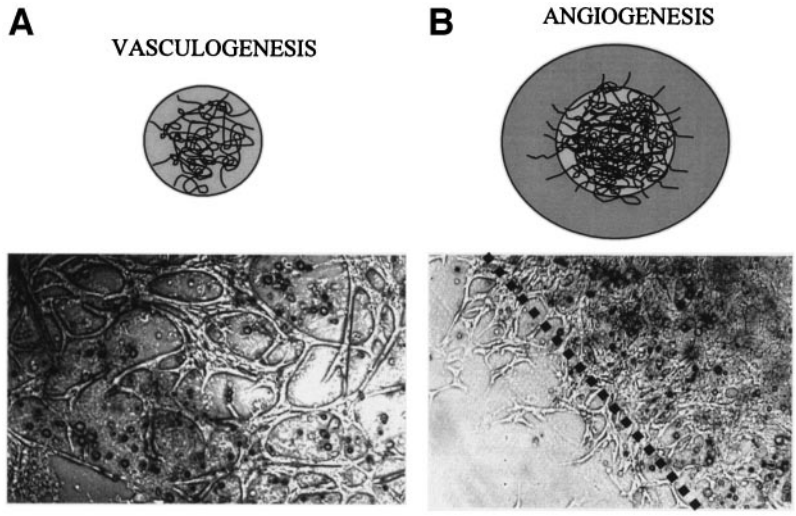

FIG. 1. Diagrammatic and morphologic illustration of 3-D model of retinal angiogenesis. *A*: Phase contrast microscopy demonstrates development of retinal microvascular endothelial cell networks and the formation of tubes within the initial 3-D matrix. *B*: A second layer of Matrigel is placed on top of the initial matrix, and this results in invasion of preformed retinal microvessels into the secondary layer. This is observed as invasion across a phase-dark line (shown as the dotted line), and tubes are counted only at the point where they cross this line.

of diabetic retinopathy, the potential modulatory role of AGEs and AGE receptors in retinal angiogenesis remains largely unknown. The current study has adopted in vitro and in vivo investigative approaches to determine whether AGEs and their receptors alter angiogenesis in the retinal microvasculature.

RESEARCH DESIGN AND METHODS

In vitro angiogenesis assay. The in vitro assay used was a novel three-dimensional model of angiogenesis that used retinal endothelial cells in an extracellular matrix gel (Matrigel; Becton Dickinson). Bovine retinal microvascular endothelial cells (RMECs) were isolated and cultured between passages 3 and 6 in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) with 20% porcine serum (Sigma) as previously described (16). RMECs were resuspended in DMEM mixed with an equal volume of Matrigel at 4°C and porcine serum to a final concentration of 20%. Forty-microliter aliquots of this suspension were plated on 3-cm petri dishes (Nunc Plasticware). After being allowed to polymerize at 37°C for 1 h, each spot was bathed in 2 ml of DMEM growth medium and 20% porcine serum. The RMECs in this environment were seen to associate and form into a complex network of endothelial tubes (Fig. 1*A*). After 24 h at 37°C, the bathing medium was aspirated and a second layer of Matrigel containing the test substances was layered over the primary culture to produce a duplex culture. On phase microscopy, a phase-dark line was seen to demarcate the interface between the primary and secondary gel layers. Again, after polymerization, each spot was bathed in 2 ml DMEM growth medium and 20% porcine serum. After a further 24 h at 37°C, endothelial sprouts were observed to invade the secondary gel layer (Fig. 1*B*). The cultures were then fixed in 4% paraformaldehyde in PBS, and the number of endothelial sprouts that had crossed the interface between the two layers around the entire circumference of each primary culture spot was counted using the phase microscope.

The nature of the endothelial tubes within this 3-D model was evaluated in fixed preparations using confocal scanning laser microscopy. The endothelial networks were visualized by combination of biotinylated isolectin B4 from *Griffonia simplicifolia* (Sigma-Aldrich, Gillingham, U.K.) at 50 ng/ml and streptavidin-Alexa 568 (Molecular Probes Europe BV, Leiden, the Netherlands). The cell nuclei were counterstained with propidium iodide (Sigma).

Cultures were also processed for transmission electron microscopy after fixation in 2.5% glutaraldehyde in 0.1 mol/l sodium cacodylate buffer (pH 7.2) containing 10 mmol/l $MgCl_2$. The fixed samples were then washed and treated with 1% osmium tetroxide for 1 h in the same buffer, dehydrated, and embedded in situ on the plastic dish in Spurr's resin for ultrathin sectioning. Transverse sections of the endothelial tubes were obtained by cutting the interface of the plastic and embedding resin.

Statistical comparisons were made between the mean number of invading sprouts/culture spot based on 10 plates per treatment, using a one-way ANOVA and Tukey-Kramer post test for multiple comparisons. Analysis was conducted on raw data. For presentation and interexperiment comparison purposes, several of the charts presented were rendered as percentages of control values.

Diabetic sera: recruitment of subjects. Sera was obtained from healthy, nondiabetic subjects (random blood glucose level <7 mmol/l) or from a range of type 2 diabetic patients attending the regional diabetic clinic, Royal Victoria Hospital, Belfast, Northern Ireland, after ethics committee consent. HbA_{1c} on two previous occasions was noted. Sera from seven individuals within the following groups were assayed together: *1*) control, nondiabetic group; *2*) well-controlled diabetic group (mean HbA_{1c} <7%); and *3*) poorly controlled diabetic group (mean HbA_{1c} >9%). Patients with albuminuria were excluded, and presence of other complications was noted. In the total patient sample, 28% had sensory neuropathy, 7% had foot ulceration, 28% had diagnosed angina, and 50% were on a drug regimen of acetylcholine esterase inhibitors, insulin, or sulfonylureas. There was no segregation in these parameters with HbA_{1c}.

The diagnosis of diabetic retinopathy in these patients was also noted and graded as background or proliferative. The criteria for inclusion in the background diabetic retinopathy group was the presence of capillary microaneurysms, dot and blot intraretinal hemorrhages, and hard exudates. The criteria for inclusion in the proliferative diabetic retinopathy group were the presence of preretinal neovascularization at the optic nerve head or elsewhere in the retina. Other noted patient data included age, duration of diabetes, BMI, random blood glucose on two previous occasions, cholesterol, triglycerides, HDLs, presence of other complications, and drug regimens.

After gaining written informed consent, ~8 ml of venous blood was taken from the antecubital fossa of each subject. The blood was collected in two polypropylene tubes, labeled with the patient's hospital number or the allocated volunteer code, and stored on ice. The blood was allowed to clot for ~30–60 min, before being centrifuged at 1,500 rpm for 20 min at 4°C. The serum was recovered and either used directly in the in vitro angiogenesis assay (above) or stored at −70°C for future use.

VEGF, AGEs, and soluble galectin-3 assay of patients sera. Sera from patients diagnosed with proliferative retinopathy (n = 7) or background retinopathy (n = 7) were analyzed for VEGF using an enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Abingdon, U.K.) according to the manufacturer's instructions. In addition, the well-characterized AGEs carboxymethyl-lysine (CML) and carboxyethyl-lysine (CEL) were analyzed from the control (nondiabetic) (n = 6), well-controlled (n = 8), and poorly controlled (n = 11) diabetic groups using gas chromatograph—mass spectrometry (GC-MS) according to previously published protocols (17). Soluble galectin-3 was quantified in control, well-controlled, and poorly controlled diabetic groups using a specific ELISA (Caltag; Bender Medsystems). Results were read immediately on a Tecan SAFIRE spectrophotometer (450-nm primary wavelength). All samples and controls were run in triplicate microwell strips, and a standard curve was calculated for each run. Statistical analysis (unpaired t test) was performed using the Graphpad PRISM statistical package.

Effect of sera from diabetic and nondiabetic patient groups on angiogenesis by retinal endothelial cells in vitro. For assay of angiogenic potential, the serum from each individual was mixed in equal amounts with Matrigel and applied as the secondary gel layer of the duplex 3-D cultures described above. Sera from six individuals within the nondiabetic, poorly controlled diabetic, and well-controlled diabetic groups were assayed. In separate experiments, serum samples from six patients with proliferative retinopathy were tested for angiogenic potential against sera from six patients with nonproliferative/background retinopathy, regardless of glycemic control in either group. In an additional experiment, pooled serum from the patients with proliferative diabetic retinopathy was assayed for angiogenic potential in

TABLE 1
Patient characteristics

	n	Age (years)	Duration of diabetes (years)	HbA_{1c} (%)	Serum cholesterol (mmol/l)	Triglycerides	HDL	BMI
Well controlled	7	62.5 ± 15.5	5.17 ± 5.27	5.48 ± 0.85	4.30 ± 0.71	2.52 ± 0.707	1.24 ± 0.1	32.2 ± 2.13
Poorly controlled	7	58.5 ± 6.44	8.0 ± 6.37	11.13 ± 1.31	4.99 ± 1.42	3.61 ± 1.16	1.23 ± 0.07	26.7 ± 0.71

Data are means ± SD.

the presence of neutralizing anti-VEGF antibody (R&D Systems) at increasing concentrations 0.001–0.1 mg/ml.

Effect of AGE-modified proteins on retinal angiogenesis in vitro. For in vitro experiments, various exogenously formed AGE-modified albumins were prepared. The modified bovine serum albumins (BSA, Fraction V [low endotoxin]; Sigma, Poole, U.K.) were added to the in vitro angiogenesis assay at concentrations ranging between 10 and 400 μg/ml. AGE-BSA was prepared from incubation in 0.5 mol/l glucose as previously described (18). CML-modified BSA (35% modification) was prepared as previously outlined (19). Glycoaldehyde-modified BSA (GA-BSA) was prepared according to Nagai et al. (20). In all cases, following dialysis against PBS, endotoxin was removed using an endotoxin-removing column (Pierce, Rockford, IL). AGE-, GA-, and CML-BSA and control, unmodified BSA were passed through separate columns three times to ensure that all contaminating endotoxin was removed.

For in vivo experiments, GA modification of mouse serum albumin (MSA) (Fraction V; Sigma) was performed and potential endotoxin contamination removed as outlined above. Analysis of the CML and CEL content of GA-MSA and native MSA was performed using GC-MS. Lysine content of the samples was analyzed by cation exchange chromatography, and the values for CML/CEL were corrected for lysine loss and expressed as mol/l CML/CEL per mol/l GA-MSA as previously reported (21).

Pretreatment with AGE receptor antibodies. Antibody to RAGE was obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). Rat monoclonal antibody to galectin-3 was collected from the tissue culture supernatants of the hybridoma M3/38. The hybridoma was obtained from the American Type Culture Collection (Manassas, VA). For AGE receptor neutralization experiments, the 3-D angiogenesis model was pretreated with RAGE, galectin-3 antibodies, or nonimmune murine IgG (200 mg/ml) for 2 h before addition of AGE or serum treatments.

Murine model of proliferative retinopathy and infusion with preformed AGEs in wild-type C57B6 and galectin-3 knockout mice. The studies outlined below adhered to the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmic and vision research and to British Home Office regulations. Oxygen-induced retinopathy was induced in C57BL/6 mice according to Smith et al. (22). In this model, 7-day-old (P7) mouse pups and their nursing dams were exposed to 75% oxygen (humidified medical-grade oxygen controlled by a PROOX oxygen controller model 110; Reming Bioinstruments, Redfield, NY) for 5 days, during which time there is vaso-obliteration and cessation of development of the central retinal capillary beds. On postnatal day 12 (P12), the mice are returned to room air, after which there is acute retinal ischemia in the avascular regions of the central retina, followed by a potent preretinal neovascular response between P15 and P21. In addition to wild-type mice, galectin-3 knockout mice ($gal3^{-/-}$), generated as previously described (23) and backcrossed to C57BL/6 for nine generations, were exposed to hyperoxia. Routinely, two pups from each litter were killed on return to room air (P12) to check for vaso-obliteration in the central retina (24).

As maternal milk production can be variable following exposure to hyperoxia, at P12 all the pups were cross-fostered to nonexposed nursing Swiss dams. Wild-type C57BL/6 and $gal3^{-/-}$ mice (also from a C57BL/6 genetic background) were randomly assigned to groups of equal size and injected intraperitoneally with either native MSA or GA-MSA (10 mg/kg) daily until P20. At P20 the mouse pups were anesthetized and perfused by intracardiac injection with 1.5 ml of saline containing 50 mg/ml of fluorescein isothiocyanate (FITC)-dextran (molecular weight [MW]: 2 × 10; Sigma-Aldrich), as previously described (24). Eyes were dissected, fixed in 4% paraformaldehyde for 18 h, and flat-mounted in a Maltese cross-configuration for image analysis.

Quantification of neovascular response in murine retina. The flat-mounted retinas were imaged at low magnification (×4) using a confocal microscope (BioRadMicroRadiance). A neovascular response was evaluated in a "blinded" fashion by a trained microscopist using a grid analysis of angiograms as outlined by Gebarowska et al. (24). Briefly, digital confocal images of fluorescein-dextran–perfused retinas were superimposed with 64-square grids, and the total area of retina—designated by the observer as either ischemic or covered by preretinal neovascularization—was estimated with the use of specially designed software (24).

All data were compared using one-way ANOVA followed by a Tukey-Kramer multiple comparisons test. Data are shown as means ± SD.

RESULTS

Patient sera: characterization of groups by clinical parameters. Levels of HbA_{1c} were significantly different between well-controlled and poorly controlled diabetic patients (5.48 ± 0.85 vs. 11.13 ± 1.31) ($P < 0.0001$). In a variety of other clinical parameters, there was no significant difference between the patient groups (Table 1). For background retinopathy versus proliferative retinopathy, there was no significant difference in any of the clinical parameters. The serum from patients in each group was tested individually to assess intergroup variation and negate interassay variation. The sera were pooled for analysis once it was clear that no patient sera produced significantly different intragroup responses (data not shown).

Quantitative analysis of soluble galectin-3 (ng/ml) of nondiabetic subjects (1.56 ± 0.98) (mean ± SD) and well-controlled (2.627 ± 1.00) and poorly controlled diabetic subjects (1.55 ± 0.94) showed no significant difference between any of the groups. Although serum CML was not significantly different between groups, CEL levels were higher in poorly controlled patients than in well-controlled patients (0.021 ± 0.001 vs. 0.057 ± 0.014 mmol/mol lysine) ($P < 0.001$).

Characterization of the 3-D model of retinal angiogenesis in vitro. Confocal microscopy revealed that endothelial sprouts invading the secondary gel layer showed characteristics consistent with active angiogenesis: At the leading edge, the cell processes expressed long filapodia, whereas downstream there was evidence of endothelial cell mitosis (Fig. 2*A* and *B*). Electron microscopy of the 3-D retinal endothelial cell cultures revealed contiguous cells joined by highly organized junctional complexes and enclosing a narrow lumen (Fig. 2*C–E*). The cells were associated with deposits of extracellular matrix material at their interface with the culture dish.

Effect of diabetic serum on in vitro angiogenesis

Normal versus diabetic and good versus poor glycemic control. A comparison of angiogenic responses produced by sera from nondiabetic and diabetic individuals (well and poorly controlled) demonstrated a highly significant suppression of angiogenesis in both diabetic groups ($P < 0.001$) (Fig. 3). The nondiabetic group showed the greatest angiogenic response, whereas the poorly controlled diabetic group had the poorest angiogenic response, reflected by the lowest mean number of sprouts (Fig. 3).

Background versus proliferative retinopathy. Sera from patients with proliferative diabetic retinopathy demonstrated a greater angiogenic response than sera from patients with background diabetic retinopathy ($P < 0.04$)

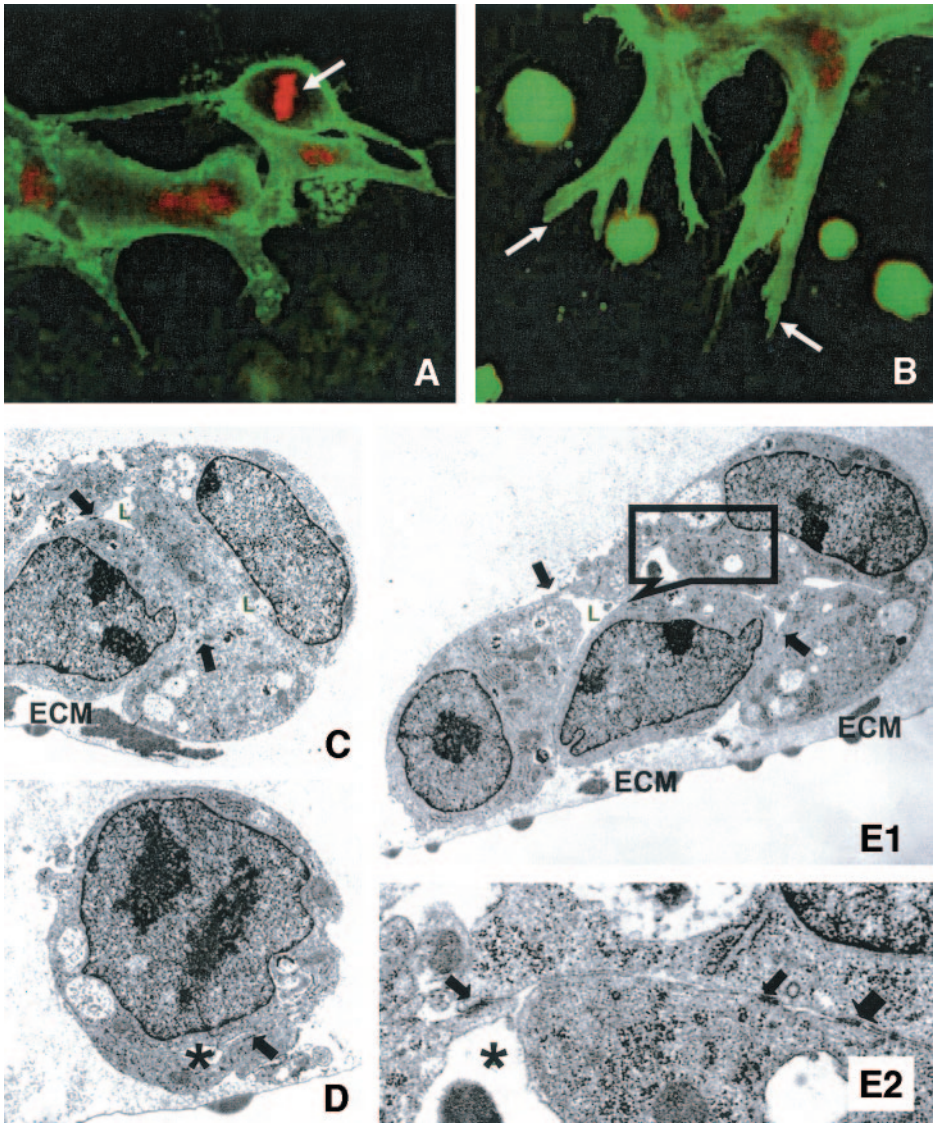

FIG. 2. Morphologic evaluation of invading retinal microvascular tubes. Isolectin staining (green) and nuclei labeling (red) evaluated by confocal scanning laser microscopy of tubes demonstrates hollow vascular tubes at the leading edge of the invading vessels. *A*: A mitotic figure is also demonstrated (arrow). The cell processes form microspike-like structures (*B*) (arrows). Original magnification ×200. Ultrastructural examination of the vessels invading the extracellular matrix (ECM) reveals interendothelial junctional complex assembly (arrows) (*C*) and lumen formation (*) (*D*) (original magnification ×9,000). There are several examples of vascular tubes showing narrow slitlike lumena (*), extensive junctional zones (arrows), and deposition of extracellular matrix (ECM) at the basal cell surface (E1). Original magnification ×9,000. Higher magnification reveals greater detail of the junctional contact as outlined by the box in E1 (E2) (original magnification ×15,000).

(Fig. 4*A*). When RMECs in the 3-D angiogenesis assay were co-exposed to VEGF-neutralizing antibody (0.001–0.1 mg/ml) and sera from patients with proliferative retinopathy, there was a stepwise suppression of the angiogenic response ($P < 0.01$) (Fig. 4*B*). This was also reflected by measurement of serum VEGF, which demonstrated that patients with proliferative retinopathy had significantly higher growth factor levels than patients with background retinopathy (211.62 ± 0.022 vs. 93.65 ± 0.013 pg/ml, $P < 0.001$).

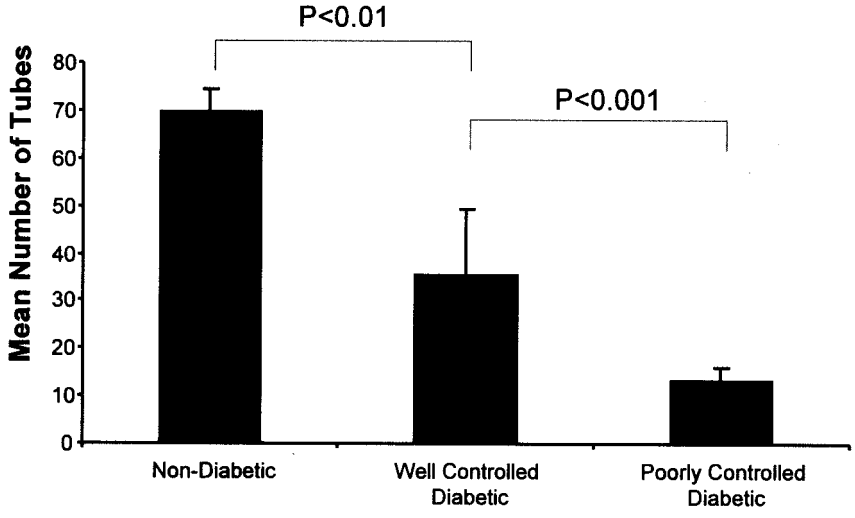

FIG. 3. Regulation of angiogenic activity in patient serum. The nondiabetic (control) group ($n = 6$) demonstrated the greatest angiogenic response, being twice that of the well-controlled group ($n = 6$) and more than fourfold that of the poorly controlled group. There was a significant difference between the angiogenic response generated in all groups, following a step-wise decline with decreased glycemic control.

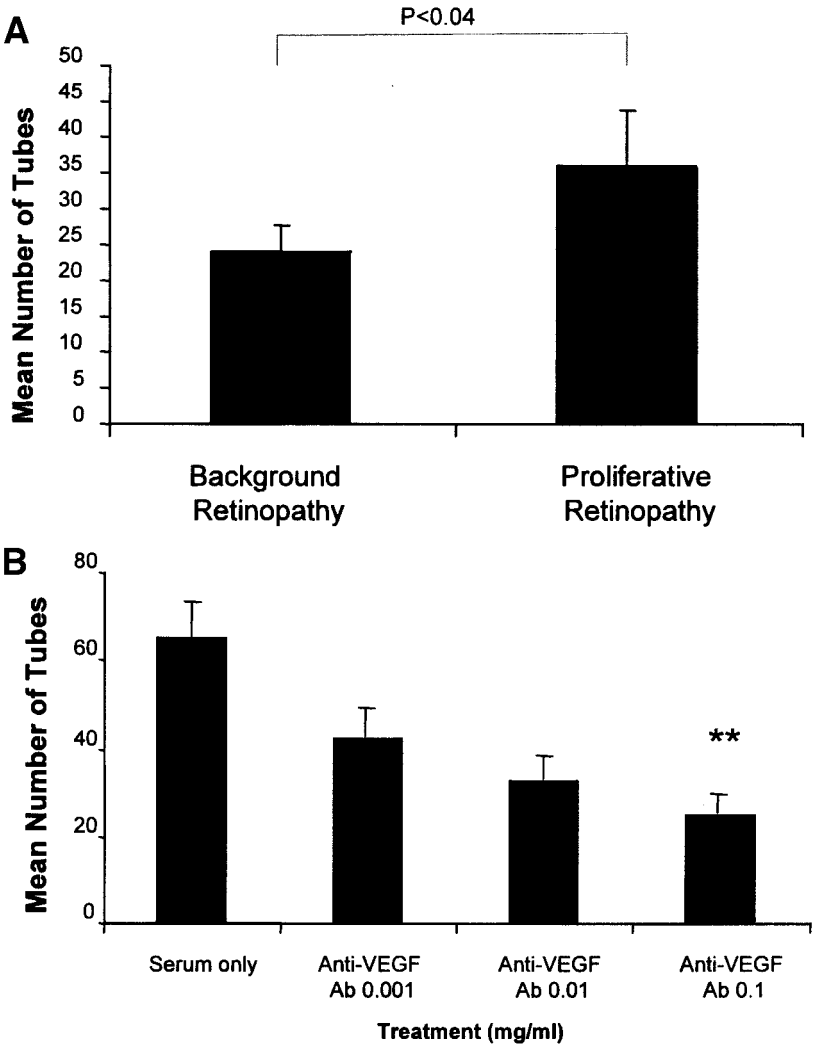

FIG. 4. Effect of serum from proliferative retinopathy patients. *A*: Serum derived from the proliferative retinopathy group showed the greatest angiogenic response, although the difference between the two groups is not quite significant. *B*: Serum from patients with proliferative retinopathy induced the greatest angiogenic effect and neutralizing anti-VEGF antibody (Ab) caused a dose-dependent decrease in angiogenic potential. **$P <$ 0.01.

Effect of AGE-modified albumins on in vitro angiogenesis. When various AGE-modified albumins were assessed in the angiogenesis assay, it was evident that these adducts suppressed angiogenic activity by RMECs when compared with native albumin controls. With increasing concentrations of CML-BSA there was a stepwise decrease in tube number in a dose-response manner ($P < 0.01$) (Fig. 5*A*). There was also a significant concentration-dependent inhibition of angiogenesis with AGE-BSA ($P < 0.001$) (Fig. 5*B*) and GA-BSA at the highest concentration ($P < 0.05$) (Fig. 5*C*).

Reversal of AGE-mediated inhibition of angiogenesis by AGE-receptor antibodies. Pretreatment of RMEC vascular tubes with an antibody to the AGE-receptor galectin-3 restored AGE-BSA–mediated suppression of angiogenesis to the extent that there was no significant difference between BSA control–treated RMECs (Fig. 6*A*). Anti-RAGE antibody also reversed AGE-BSA–induced inhibition, although this pretreatment significantly enhanced the angiogenic response when compared with control subjects ($P < 0.001$) (Fig. 6*B*). Galectin-3 antibodies also reversed diabetic serum–induced suppression of angiogenesis, restoring tube invasion to levels comparable to nondiabetic control subjects (Fig. 6*C*).

AGEs inhibit preretinal neovascularization in vivo. As previously demonstrated (22,24), exposure of neonatal mice to hyperoxia for 5 days, followed by a return to room air for a further 9 days, caused a potent, ischemia-driven preretinal neovascular response at P20 (Fig. 7*A*). When wild-type mice were injected intraperitoneally with GA-MSA from P12 to P20 ($10\ mg \cdot kg^{-1} \cdot day^{-1}$) and the retinal microvasculature was assessed at P20, there was a significant increase in the area of ischemia in the inner retina when compared with native MSA controls ($P < 0.01$) (Fig. 7*B* and *D*). Also, despite having greater areas of nonperfusion, there was a GA-MSA–mediated reduction in preretinal neovascularization ($P < 0.001$) (Fig. 7*E*).

AGE-mediated inhibition of preretinal neovascularization is prevented by absence of galectin-3. OIR in gal3$^{-/-}$ mice produced no significant difference in retinal ischemia compared with wild-type mice at P20 (compare Fig. 7*A* and *C*; Fig. 7*D*). However, mice that lacked galectin-3 showed a greater overall angiogenic response ($P < 0.05$). Exposure of gal3$^{-/-}$ mice to GA-MSA failed to induce a significant increase in retinal ischemia or a decrease in preretinal neovascularization when compared with MSA controls (Fig. 7*E*).

DISCUSSION

The current study has sought to determine the pro- or anti-angiogenic effect of diabetic serum in parallel with

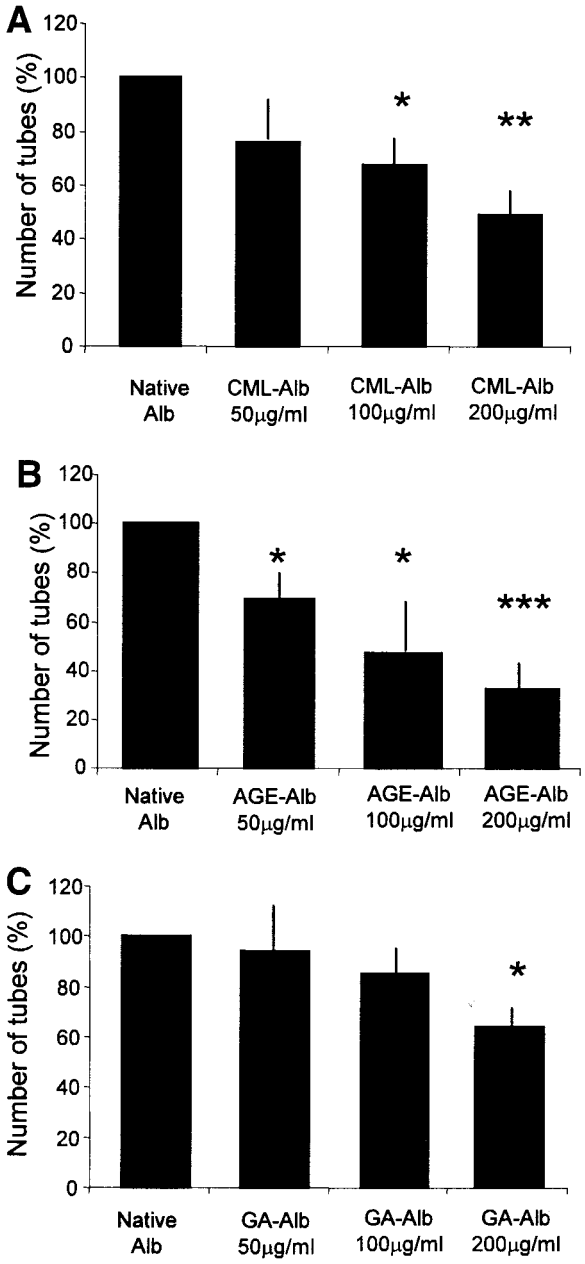

FIG. 5. Albumin modified by advanced glycation suppresses angiogenesis. CML-BSA (*A*), AGE-BSA (*B*) and GA-BSA (*C*) cause a stepwise decrease in retinal angiogenesis in vitro. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Data represent means values ± SD from three independent experiments.

defined AGE exposure. Evidence is presented that indicates a suppression of retinal angiogenesis within the diabetic state and implicates AGEs and AGE receptor interactions in this phenomenon; the angiogenic effects of AGEs were further demonstrated in a murine model of ischemia-induced neovascularization.

The in vitro retinal angiogenesis assay adopted in the current investigation provides many advantages over conventional approaches that dissect individual processes, such as endothelial cell proliferation, matrix invasion, migration, and tube formation. Individually, these parameters are useful indicators of angiogenic activity, but the 3-D system we utilized induces sprouting, migration, matrix invasion, and proliferation by retinal capillary endothelial cells from within preformed tubular networks. This system thus replicates all of the major components of angiogenesis in vivo and provides a highly relevant model for in vitro studies of pro- and anti-angiogenic agents. Indeed, the morphologic characterization of the model further confirms that angiogenesis within the model closely mimics retinal angiogenesis in vivo. The tubular conformation of the cell aggregates within the endothelial networks was confirmed by electron microscopy, and all profiles revealed lumen formation and elaborate cell junctions. Furthermore, during endothelial sprouting and invasion of the secondary gel layer, the leading edge of the cell processes expressed long filapodia similar to those seen at the angiogenic front during developmental angiogenesis in the neonatal retina (25).

Using the above system, it was found that diabetic sera inhibited angiogenesis relative to nondiabetic control sera and that serum from poorly controlled diabetic patients inhibited angiogenesis to a greater extent than that from their well-controlled counterparts. This suggests that diabetes reduces angiogenic potential in the retinal microvasculature and that the phenomenon is correlated with the level of glycemic control. This is a novel and important finding since it suggests that, apart from inactive proliferative retinopathy, the angiogenic potential of retinal microvascular endothelial cells is significantly compromised by the diabetic state. In other organ systems, there is a widely recognized inhibition of angiogenesis during diabetes (4,8), and it seems likely that in the early phases of diabetic retinopathy there is also an anti-angiogenic state within the retinal vasculature, despite evidence of increased endothelial cell turnover. Sharma et al. (26) demonstrated that retinal microvascular endothelium shows a threefold increase in cell replication in diabetic rats compared with nondiabetic controls. However, the pattern of labeling with ^{3}H-thymidine in retinal vascular digests led the authors to conclude that the increased endothelial cell turnover represented increased replacement rather than proliferation (26), a conclusion that was confirmed by the finding that there is accelerated cell death in the retinal microvasculature during diabetes (27).

Serum from patients with proliferative retinopathy showed a greater angiogenic effect than that from patients with background retinopathy, suggesting that in the proliferative phase of the disease there were sufficiently high levels of factor(s) that overcome the anti-angiogenic nature of the diabetic state. Undoubtedly VEGF is one such factor, and the present study has shown it to be responsible for a significant measure of the angiogenic potential in serum from patients with proliferative retinopathy. High levels of VEGF have been identified in ocular fluids of diabetic patients (28), and while it appears that serum VEGF does not influence intraocular concentrations (29), it is not known whether the converse is also true. Although two reports have suggested no correlation between retinopathy and serum VEGF (30,31), other studies have indicated significantly higher levels of VEGF in the sera of patients with proliferative compared with background retinopathy that correlated with HbA_{1c} (32). Moreover, serum VEGF levels may be reduced after pan-retinal photocoagulation and regression of neovascularization (33). It is not clear whether VEGF release from ischemic

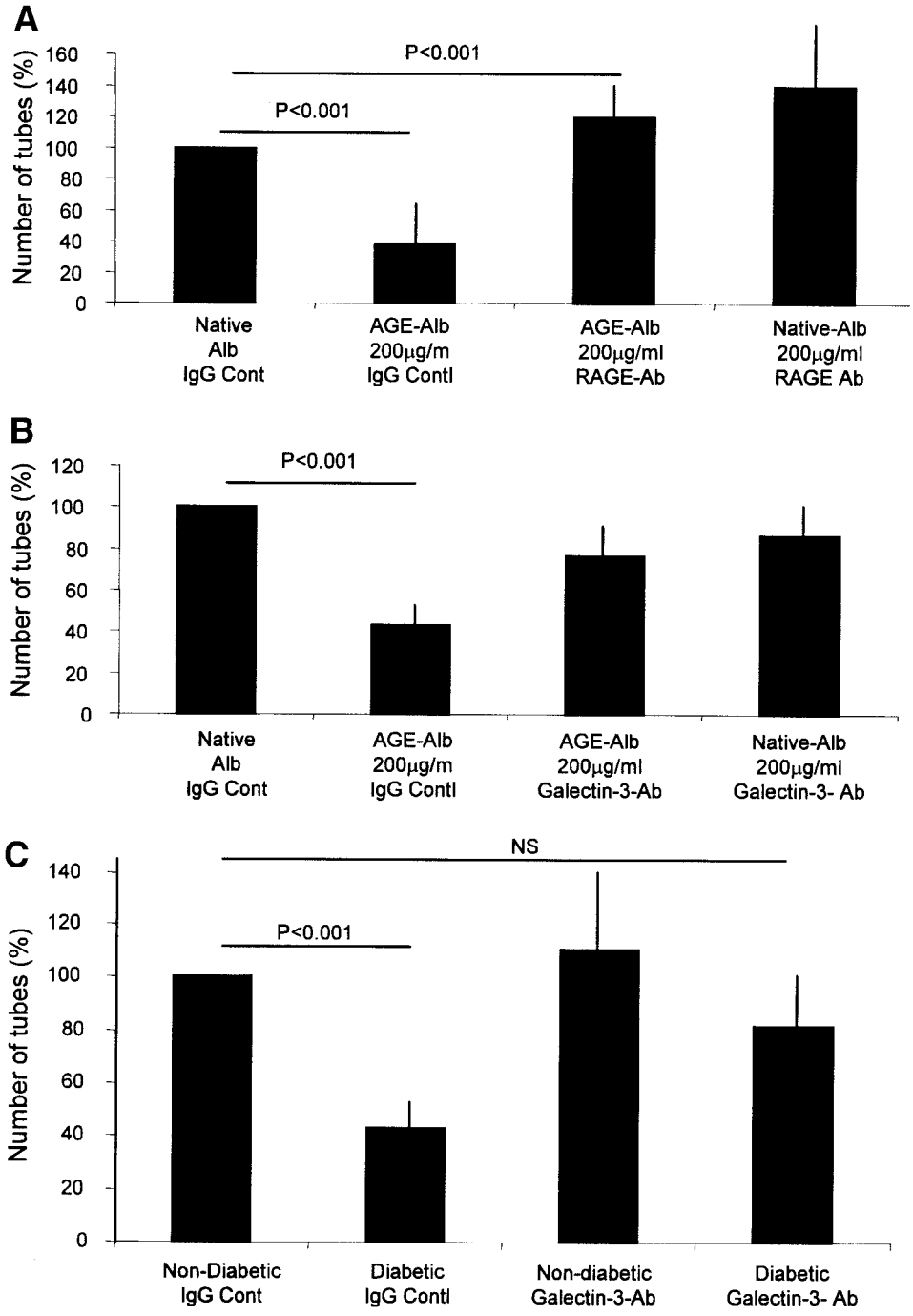

FIG. 6. AGE receptor neutralization restores AGE-mediated suppression of retinal angiogenesis in vitro. *A*: Pretreatment of RMEC vascular tubes with an antibody to the AGE receptor galectin-3 restored AGE-BSA–mediated suppression of angiogenesis to the extent that there was no significant difference between BSA control–treated RMECs. *B*: Anti-RAGE antibody also reversed AGE-BSA–induced inhibition, although this pretreatment significantly enhanced the angiogenic response when compared with control subjects ($P < 0.001$). *C*: Galectin-3 neutralization also reversed diabetic serum–induced suppression of angiogenesis, restoring tube invasion to levels comparable to nondiabetic control subjects.

retina is sufficient to significantly raise the plasma concentration of the peptide or whether the release of VEGF or some other factor is able to trigger a more global response. In either case, it was evident in the present study that pro-angiogenic concentrations of VEGF may be present in the circulation of patients with proliferative diabetic retinopathy. It is unlikely that relative VEGF concentrations were responsible for the greater angiogenic potential of serum from nondiabetic and well-controlled diabetic patients in the present study. Rather, we would suggest that the higher HbA_{1c} in the poorly controlled patients reflected inhibitory concentrations of AGEs.

Several clinical studies have reported that the levels of AGEs in diabetic serum (34) or tissues (35) are consistently greater than in nondiabetic counterparts. As a complement to studying patient sera, the current study has shown that various AGE adducts may have a significant inhibitory effect on the angiogenic potential of retinal microvascular endothelial cells in vitro and also in a murine model of proliferative retinopathy. This agrees with previous studies that have indicated AGE-mediated suppression of angiogenesis in a diabetic limb ischemia model (8) and suggests that the retinal microvasculature may suffer diabetes-related depression of angiogenic potential comparable to other vascular beds. Some reports suggest a pro-angiogenic AGE effect, at least after acute exposure (13,14,36–38), although more long-term exposures to these adducts appear to cause reduced cell

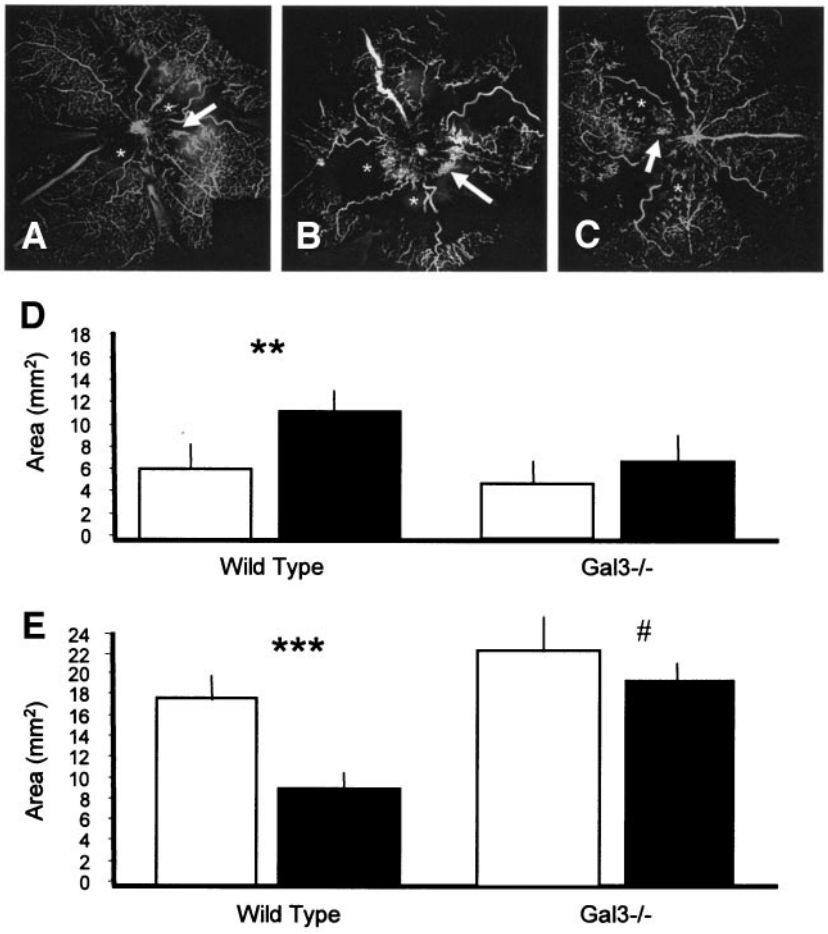

FIG. 7. AGEs increase retinal ischemia and reduce neovascularization in an in vivo model. *A*: The hyperoxia-induced retinopathy model results in retinal ischemia (*) at P20, which drives a neovascular response depicted by hyperfluorescent preretinal fronds (arrow). *B*: When wild-type mice are infused with GA-MSA between P12 and P20, the retinal microvasculature shows more ischemia when compared with native-MSA controls assessed at P20, and there was a significant increase in the area of ischemia (*) in the inner retina but less neovascularization (arrow) when compared with native MSA controls. *C*: Gal3$^{-/-}$ mice infused with GA-MSA fail to show the extensive ischemia evident in the wild-type mice exposed to this modified albumin. *D*: Quantification of retinal ischemia in wild-type mice shows that GA-MSA (■) induces enlarged areas of ischemia (indicative of suppressed intraretinal angiogenesis) at P20 when compared with native MSA controls (□). In gal3$^{-/-}$ mice, there is no difference between MSA control and GA-MSA treatment. For all experiments, there were a minimum of 10 mice per group, and data are presented as means ± SD. ***P* < 0.01 between GA-MSA and native-MSA treatments. *E*: In wild-type mice, exposure to GA-MSA causes a significant suppression of preretinal neovascularization. The gal3$^{-/-}$ neonatal mice show a greater overall angiogenic response, and exposure to GA-MSA fails to alter preretinal neovascularization when compared with wild-type. For all experiments, there were a minimum of 10 mice per group, and data are presented as means ± SD. ***P* < 0.01 and ****P* < 0.001 between GA-MSA and native-MSA treatments; #*P* < 0.05 between wild-type and gal3$^{-/-}$.

viability and impaired proliferative responses (7). The observed disparity may also be due to differences within the in vitro systems used and the time frame of studies. It seems clear that AGEs can upregulate VEGF in many cell types, including an autocrine stimulatory role in endothelial cells (38). Nevertheless, conflicting reports on AGE-induced RMEC proliferative changes are evident (39,40), but it is restrictive and possibly misleading not to address the complex angiogenic process as several interdependent cell events.

AGE-receptors can modulate many cell responses, and the current study has demonstrated that antibodies to both galectin-3 (also referred to as AGE-R3 [41]) and RAGE can reverse AGE-mediated suppression of retinal angiogenesis. RAGE antibodies actually enhanced angiogenic activity, although the underlying mechanism for this remains unclear. Previous studies have shown that inhibition of RAGE-AGE interactions can restore diabetes-related impairment of angiogenesis during wound healing in mice (9). We have now demonstrated a comparable mechanism with galectin-3, and it is interesting that neutralization or absence of this AGE receptor reversed the inhibition of retinal angiogenesis by diabetic sera or exogenous AGEs. The importance of galectin-3 as an AGE receptor has been previously demonstrated (42–44), and the current study suggests that this protein could play a significant role in AGE-related pathophysiology during diabetic retinopathy, although no differences in the soluble fragment of this protein were demonstrated between the various patient groups. However, the pro-angiogenic effects of galectin-3 inhibition/deletion in the AGE-exposed retinal microvasculature may be both disease- and tissue-specific: Independent of AGE-binding, galectin-3 has been shown to promote cell adhesion/invasion (45), arrest apoptosis (46), and increase angiogenesis in various cancers and endothelial cells in vitro (47). These studies suggest that galectin-3 is not anti-angiogenic per se and that its inhibition would not be expected to have a generally pro-angiogenic outcome in a nondiabetic environment.

Also, Pugliese et al. (44) have demonstrated that galectin-3 shows a contrasting regulatory response to RAGE when renal cells are exposed to AGEs, indicating that galectin-3 could be protective in diabetic nephropathy. It should also be considered that galectin-3 has been only relatively recently described as a putative AGE receptor, and it is well established that this protein has many roles in cell adhesion, inflammatory responses, cell differentiation, and chemoattraction (48). Both in vitro and in vivo systems used in the current study are devoid of any overt inflammatory involvement. Nevertheless, it remains possible that neutralization or genetic depletion of galectin-3 could possibly exert effects on retinal angiogenesis that are additional to or distinct from AGE binding.

It is a common perception that during diabetic retinopathy there is a generalized pro-angiogenic state that culminates with preretinal neovascularization during the proliferative phase of the disease. If this is true, it suggests that during diabetes the retina is highly unique among the body's other vascular beds, which all show a depressed angiogenic potential. However, detailed evaluation of the

histopathological sequelae of vascular degeneration during background diabetic retinopathy provides strong evidence of a progressive endotheliopathy leading to closure and death of retinal capillaries (26,27,49,50). It is only when there is considerable nonperfusion of the retina that a potent neovascular response occurs in response to acute upregulation of angiogenic growth factors as a direct result of retinal ischemia (51). Inhibition of angiogenic mechanisms during diabetes may constitute an endotheliopathy that suppresses intraretinal vascular repair, thereby significantly contributing to ischemia as diabetic retinopathy progresses.

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