The Role of Advanced Glycation End Products in Retinal Microvascular Leukostasis

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PURPOSE. A critical event in the pathogenesis of diabetic retinopathy is the inappropriate adherence of leukocytes to the retinal capillaries. Advanced glycation end-products (AGEs) are known to play a role in chronic inflammatory processes, and the authors postulated that these adducts may play a role in promoting pathogenic increases in proinflammatory pathways within the retinal microvasculature.

METHODS. Retinal microvascular endothelial cells (RMECs) were treated with glycoaldehyde-modified albumin (AGE-Alb) or unmodified albumin (Alb). NFκB DNA binding was measured by electromobility shift assay (EMSA) and quantified with an ELISA. In addition, the effect of AGEs on leukocyte adhesion to endothelial cell monolayers was investigated. Further studies were performed in an attempt to confirm that this was AGE-induced adhesion by co-incubation of AGE-treated cells with soluble receptor for AGE (sRAGE). Parallel in vivo studies of nondiabetic mice assessed the effect of intraperitoneal delivery of AGE-Alb on ICAM-1 mRNA expression, NFκB DNA-binding activity, leukostasis, and blood–retinal barrier breakdown.

RESULTS. Treatment with AGE-Alb significantly enhanced the DNA-binding activity of NFκB ($P = 0.0045$) in retinal endothelial cells (RMECs) and increased the adhesion of leukocytes to RMEC monolayers ($P = 0.04$). The latter was significantly reduced by co-incubation with sRAGE ($P < 0.01$). Mice infused with AGE-Alb demonstrated a 1.8-fold increase in ICAM-1 mRNA when compared with control animals ($P < 0.001$, $n = 20$) as early as 48 hours, and this response remained for 7 days of treatment. Quantification of retinal NFκB demonstrated a threefold increase with AGE-Alb infusion in comparison to control levels (AGE Alb versus Alb, 0.23 vs. 0.076, $P < 0.001$, $n = 10$ mice). AGE-Alb treatment of mice also caused a significant increase in leukostasis in the retina (AGE-Alb versus Alb, 6.89 vs. 2.53, $n = 12$, $P < 0.05$) and a statistically significant increase in breakdown of the blood–retinal barrier (AGE Alb versus Alb, 8.2 vs. 1.6 $n = 10$, $P < 0.001$).

CONCLUSIONS. AGEs caused upregulation of NFκB in the retinal microvascular endothelium and an AGE-specific increase in leukocyte adhesion in vitro was also observed. In addition, increased leukocyte adherence in vivo was demonstrated that was accompanied by blood–retinal barrier dysfunction. These findings add further evidence to the thinking that AGEs may play an important role in the pathogenesis of diabetic retinopathy. (*Invest Ophthalmol Vis Sci.* 2003;44:4457–4464) DOI:10.1167/iovs.02-1063

The formation and accumulation of advanced glycation end-products (AGEs)[1] is one of several pathogenic mechanisms that may contribute to diabetic microvasculopathy. AGEs can form on the amino groups of proteins, lipids, and DNA through several complex pathways, including nonenzymatic glycation by glucose and reaction with ascorbate, metabolic intermediates, and reactive dicarbonyl intermediates. These reactions modify the structure and function of proteins and may lead to formation of complex cross-links.[2] Metal-catalyzed oxidative reactions also give rise to a group of AGEs, classified as glycoxidation products (e.g., N^{ϵ}-(carboxymethyl)lysine [CML] or N^{ϵ}-(carboxyethyl)lysine [CEL])[3,4] that also accumulate on macromolecules with aging and at an increased rate in diabetes.[5]

AGEs can induce a range of pathogenic effects in retinal microvascular endothelium in vitro, many of which are mediated through AGE-receptors.[6–8] In in vivo systems, however, the role of AGEs in diabetic retinopathy continues to remain equivocal. AGEs are known to accumulate in the neural retina and vascular cells of diabetic animals[9,10] where they appear to initiate pathophysiological changes in retinal microvascular function.[11] In addition, the AGE inhibitor, aminoguanidine, can attenuate formation of retinopathic lesions in diabetic animal models.[12–16] A critical early event in the pathogenesis of diabetic retinopathy is leukocyte adhesion to the diabetic retinal vasculature. The process is mediated in part by upregulation of intercellular adhesion molecule (ICAM)-1 by the retinal microvascular endothelium[17] and contributes to blood–retinal barrier breakdown and capillary nonperfusion.[18] Recent data have pointed to the role of leukocyte adhesion in the production of retinal disease, and diabetic retinopathy is now a recognized inflammatory disease. Significantly, the ICAM-1 and VCAM-1 genes are controlled by multiple binding sites for transcription factors, including NF-κB, which is closely linked to AGE-mediated generation of oxidative stress.[19–21]

The role of AGEs in modulation of proinflammatory responses during the development of diabetic retinopathy remains unknown. In the present study we investigated the effect of the AGE-modified proteins on the expression of adhesion molecule ICAM-1, transcription factor NFκB and leukocyte adhesion in vitro and in vivo.

METHODS

Preparation of AGE-Modified Proteins

AGE-modification of bovine serum albumin (BSA) or mouse serum albumin (MSA; fraction V; Sigma-Aldrich, Poole, UK) was performed according to the protocols previously described by Nagai et al.[22] Briefly, albumin (2 mg/mL) was incubated with 33 mM glycoaldehyde at 37°C for 7 days in phosphate-buffered saline (PBS; pH 7.4). After dialysis against PBS, endotoxin was removed using an endotoxin-separation column (Pierce, Inc., Rockford, IL). Glycoaldehyde-modified albumin (AGE-Alb) and native albumin (Alb) were passed through separate columns three times to ensure that all contaminating endotoxin had been removed. Aliquots were tested for endotoxin by an independent company (Endosafe; Charles River Laboratories, Wilmington, MA).

In addition, BSA which had been modified to yield CML specifically was characterized. The CML-BSA was kindly prepared by Suzanne Thorpe (University of South Carolina). Briefly, 10% and 35% modified albumin was prepared by reacting different ratios of glyoxylic acid (COOHCHO) with BSA in the presence of sodium cyanoborohydride.

Analysis of the CML and CEL content of AGE-Alb and native albumin was performed by Suzanne Thorpe, using gas chromatography mass spectrometry (GC-MS). The specificity and degree of modification of the 10% and 35% modified BSA were quantified with GC-MS analysis as well.

Lysine content of the samples was analyzed by cation exchange chromatography, and the levels of CML and CEL were corrected for lysine loss and expressed as moles CML or CEL per mole BSA.[23]

In Vitro Experiments

AGE Treatment of Endothelial Cells. HUVECs or bovine retinal microvascular endothelial cells (RMECs, passages 2–4) were isolated and cultured as described previously.[24] RMEC monolayers were cultured in phenol red–free DMEM containing 10% fetal calf serum, to which AGE-Alb at various concentrations (150–400 μg/mL) was added. Control cells were grown in the presence of native albumin at the same concentration for 1 to 24 hours.

Nuclear Extraction from AGE-Treated Cells and Measurement of NFκB by EMSA. Nuclear protein was extracted from AGE-treated cells or control cells and assessed for NFκB DNA binding activity by electromobility shift assay (EMSA), as previously described by Digman et al.[25] Briefly, cells were washed with ice-cold PBS and lysed in hypotonic buffer followed by centrifugation at 14,000 g for 10 minutes at 4°C. The pellet was resuspended in hypotonic buffer with 0.01% vol/vol Igepal and incubated for 10 minutes on ice followed by centrifugation. The pellet was resuspended in nuclear lysis buffer for 15 minutes on ice and after centrifugation the supernatant containing the nuclear extract was collected and stored at −70°C. A ^{32}P-labeled probe for NFκB (5′ AGTTGAGGGGACTTTCCCAGGC 3′; Promega, Madison, WI) was prepared and incubated with protein for 45 minutes at 65°C. The radiolabeled mixture was separated on 40% bis-acrylamide gels, and bands on the gels were visualized after developing overnight at −80°C on autoradiograph film (Eastman Kodak, Rochester, NY).

Aliquots of nuclear protein were also assayed for p65 activity using quantitative ELISA as described later.

In Vitro Adhesion of Isolated Leukocytes. Fresh heparinized human blood from a healthy volunteer was collected, and the peripheral blood mononuclear cells (MNCs) were isolated as previously described by Pertoft et al.[26] The MNCs were used immediately for the endothelial cell–leukocyte adhesion assay. An in vitro adhesion assay was performed using human umbilical vein endothelial cells (HUVECs) and MNCs treated for 4 hours with AGE-Alb (100 μg/mL). The MNCs were labeled with a lipophilic fluorescent probe (Cell Tracker CM-DiI; Molecular Probes, Eugene, OR) before incubation on endothelial cells. After they were washed twice, the DiI-labeled cells (5 × 10⁵ cells/mL) were added to the confluent monolayers of endothelial cells for 30 minutes at 37°C. The nonadherent cells were removed with prewarmed medium, and the fluorescent attached cells

were quantified with a 96-well microplate reader (Molecular Devices, Sunnyvale, CA).

Preparation of Soluble RAGE. The soluble form of RAGE (sRAGE) was prepared in a baculovirus expression system (6× His Expression and Purification Kit; BD PharMingen, San Diego, CA) with Sf9 insect cells. Serum-free medium containing sRAGE with 6× histidine at the N-terminal was subjected to purification with Ni-NTA agarose gel. The final product produced a single band of ~40 kDa in SDS-PAGE. Endotoxin content in the sample was measured by an amebocyte lysate assay (Limulus, E-Toxate; Sigma-Aldrich) and found to be below detectable level (<0.02 ng/mL).

Effect of Co-incubation with sRAGE. An in vitro leukocyte adhesion assay was performed exactly as just described. The effect of co-incubation of sRAGE (1500 μg/mL) with AGE-Alb (100 μg/mL) on leukocyte adhesion to endothelial cells was monitored. The same concentration of nonimmunized mouse IgG (Sigma-Aldrich) was used as a control protein to test for the effect of a generic protein.

In Vivo Experiments

Animals and Anesthesia. Twenty-gram male C57/BL6 mice were obtained from Charles River Laboratories. All animal experiments were approved by the Massachusetts Eye and Ear Infirmary Animal Care and Use Committee and conformed to the Association for Research in Vision and Ophthalmology guidelines. Before all experimental manipulations, the mice were anesthetized with an intramuscular injection of 25 mg/kg of ketamine hydrochloride (Parke-Davis, Morris Plains, NJ) and 4 mg/kg of xylazine hydrochloride (Phoenix Pharmaceuticals, St. Joseph, MO).

AGE Treatment of Mice. AGE-treated mice received 10 mg/kg AGE-Alb or native albumin every day for seven consecutive days by interperitoneal injection. All proteins were passed through an endotoxin-removing column (Pierce, Inc.) and the absence of contaminating endotoxin was confirmed by an independent company (Endosafe; Charles River Laboratories). Endotoxin free PBS (Sigma-Aldrich) was used for any necessary dilution of AGE samples before injection.

Real-Time PCR Quantification of ICAM-1 mRNA Expression in Retina. *Mouse Retinal RNA Extraction.* Groups of mice were killed as just described, the eyes enucleated, and the retina dissected away from the posterior eye cup and placed in a stabilization reagent (RNA-Later; Ambion, Inc. Austin, TX) at 4°C. Total retinal RNA was isolated with extraction reagent (TRIzol Reagent; Invitrogen-Gibco, Paisley, Scotland, UK) according to manufacturers' instructions. Briefly, one retina was mixed with 1 mL of extraction reagent at room temperature. Retinas were homogenized using a plastic pestle (Geno-Technology Inc., Maplewood, MO) attached to a handheld drill for three 15-second bursts, and the lysate was allowed to sit at room temperature for 10 minutes to allow nucleoprotein dissociation. The lysate was loaded into a shredder (Qiashredder; Qiagen Inc., Valencia, CA) to aid homogenization, followed by centrifugation at 12,000g for 5 minutes. The supernatant was removed to an RNase free tube (Eppendorf, Fremont, CA), and 200 μL chloroform was added and vortexed to mix. After incubation at room temperature for 10 minutes, phase separation was performed by centrifugation at 12,000g for 15 minutes at 4°C. The upper aqueous phase containing the RNA was carefully removed to an RNase-free tube and the RNA precipitated in 500 μL isopropanol for 15 minutes followed by centrifugation at 12,000g at 4°C. The RNA pellet was washed twice with 75% ethanol and resuspended in 25 μL of diethyl pyrocarbonate (DEPC) water. RNA integrity and quality was confirmed by analysis by 260:280-nm ratio and visualization of ribosomal 28S and 18S RNA bands on a 0.5% agarose gel. cDNA was synthesized from 2 μg total retinal RNA using a reverse transcriptase cDNA synthesis kit (Superscript II; Invitrogen-Gibco) according to the manufacturer's instructions on an automated system (GeneAmp PCR System 9700; Applied Biosystems, Foster City, CA). cDNA was diluted fivefold before PCR amplification.

Real-Time PCR. Real-time PCR analysis was performed using the fluorogenic probe-based 5′ exonuclease assay (*Taq*man, Applied Biosystems) on an automated sequence detection system (model 7700;

Applied Biosystems) according to the manufacturers' instructions. Reactions were performed in a 50-μL volume of a master mix (*Taq*man Universal PCR Master Mix; Applied Biosystems) with the following sets of primers and probes for detection of ICAM-1 and RPL32: mRPL32 probe CCTCTGGTGAAGCCCAAGATCGTCA, forward primer TCATGGCTGCCCTCCG, reverse primer TGACTGGTGCCTGATGAACTTCT; mICAM-1 probe TCCGTGCAGGTGAACTGTTCTTCCTCA, forward primer AGGTATCCATCCATCCCAGAGA, reverse primer GAGCTCATCTTTCAGCCACTGA.

A quantitative RT-PCR methodology (*Taq*man; Applied Biosystems) was used to measure the retinal ICAM-1 gene copy number, which was normalized to the ribosomal protein L32 mRNA copy number.[27] Linear standard curves were created during each amplification using 10^2 to 10^8 copies of ICAM-1 or RPL32 plasmids (gifts from Iain Campbell, Scripps Research Institute, La Jolla, CA). Control amplification (no probe and no template) samples were included in each reaction to set baseline and threshold levels.

Measurement of Activation of Retinal NFκB

Preparation of Nuclear Extracts. Eyes were enucleated after seven daily intraperitoneal injections of 10 mg/kg AGE (GA-Alb) or nonglycated albumin (Alb, 10 mg/kg), and the retinas were removed and snap frozen. Nuclear extraction of retinal protein was performed as described previously. Briefly, retinas were snap frozen and stored at $-70°$C. Pooled retinas were homogenized with a mechanical homogenizer in five pellet volumes of Buffer A (20 mM Tris [pH 7.6], 10 mM KCl, 0.2 mM EDTA, 20% [by vol] glycerol, 1.5 mM MgCl$_2$, 2 mM dithiothreitol [DTT]), 1 mM Na$_3$VO$_4$ and protease inhibitors; Complete; Roche Diagnostics, Mannheim, Germany). Nuclei were pelleted (2500g, 10 minutes) and resuspended in two pellet volumes of Buffer B (identical with Buffer A except that KCl was increased to 0.42 M). Nuclei debris was removed by centrifugation (15,000g, 20 minutes), and the supernatant was dialyzed against one change of buffer Z (20 mM Tris-HCl [pH 7.8], 0.1 M KCl, 0.2 mM EDTA, and 20% glycerol) for at least 3 hours at 4°C in dialysis cassettes (Dialyze Z; Pierce, Inc.). Protein concentration was measured with the bicinchoninic acid (BCA) assay.

Quantification of NF-κB Activation. NF-κB activation was analyzed with a transcription factor assay kit (trans-AM NF-κB/p65; Active Motif North America, Carlsbad, CA) according to the manufacturer's instructions. Briefly, 2 mg of the retinal nuclear extracts or bovine retinal endothelial cell extracts (prepared as listed earlier) were incubated with an oligonucleotide containing the NF-κB consensus site (5'-GGGACTTTCC-3') bound to a 96-well plate. After extensive washes, the NF-κB complexes bound to the oligonucleotide were incubated with an antibody directed against the NF-κB p65 subunit at a dilution 1:1000. After washes, the plates were subsequently incubated with a secondary antibody conjugated to horseradish peroxidase (1:1000), and the peroxidase reaction was quantified at 450 nm with a reference wavelength of 655 nm. Results are expressed in absorbance units corrected for interference at the reference wavelength.

Evan Blue Leakage Assay for Quantification of Inner Blood–Retinal Barrier

Mice were treated with AGEs as described earlier and processed for Evans blue leakage assay for detection of breakdown of the inner blood–retinal barrier, exactly as described previously by Xu et al.[28] and Qaum et al.[29] Evans blue dye was injected into the bloodstream of mice treated with AGE-Alb or native albumin through the tail vein and allowed to circulate and bind to plasma albumin.

Briefly, animals were anesthetized, and Evans blue was injected through the tail vein over 10 seconds at a dosage of 45 mg/kg. Immediately after Evans blue infusion, the mice turned visibly blue, confirming their uptake and distribution of the dye. After the dye had circulated for 60 minutes, the chest cavity was opened, and the mice were perfused for 2 minutes through the left ventricle at 37°C with 0.05 M (pH 3.5) citrate-buffered paraformaldehyde (1% wt/vol; Sigma-Aldrich). The perfusion was at a physiological pressure of 120 mm Hg.

Immediately after perfusion, both eyes were enucleated and bisected at the equator. The retinas were carefully dissected and thoroughly dried in a concentrator (Speed-Vac; Thermo Savant, St. Paul, MN) for 5 hours. Evans blue was extracted by incubating each retina in 65 μL formamide (Sigma-Aldrich) for 18 hours at 70°C. The supernatant was filtered through tubes (Ultrafree-MC; 30,000 NMWL; Millipore, Bedford, MA) at 3000g, and the filtrate was used for triplicate spectrophotometric measurements (Du-640; Beckman Instruments, Fullerton, CA). Each measurement occurred over a 5-second interval, and all sets of measurements were preceded by known standards. The background-subtracted absorbance was determined by measuring each sample at both 620 nm, the absorbance maximum for Evans blue in formamide, and 740 nm, the absorbance minimum. The concentration of dye in the extracts was calculated from a standard curve of Evans blue in formamide and normalized for retina dry weight. Blood–retinal barrier breakdown was calculated with the following equation, with results expressed in microliters plasma $\times$ grams retina dry weight $\times$ hours

$$\frac{\text{Evans blue } (\mu g)/\text{retina dry weight (g)}}{\text{time-averaged Evans blue concentration}\ (\mu g/\text{plasma } \mu L)/\text{circulation time (hr)}}.$$

Results were expressed as the percentage of native Alb control levels.

Quantification of Retinal Leukostasis

With mice under deep anesthesia, the chest cavity was carefully opened, and a 14-gauge perfusion cannula was introduced into the left ventricle into the ascending aorta. Drainage was achieved by cutting the edge of the right atrium. Animals were perfused with PBS (250 mL/kg body weight, 4 mL) at physiologic pressure (flow rate 0.2 mL/sec) to remove erythrocytes and nonadherent leukocytes from the vasculature. This was followed by perfusion with FITC-conjugated concanavalin A lectin (20 μg/mL in PBS [pH 7.4], 5 mg/kg, 4 mL) to label the adherent leukocytes and the vascular endothelial cells. Residual unbound lectin was removed by a repeat PBS perfusion. Eyes were enucleated and the retinas carefully removed and flatmounted, and leukocyte adherence to vessel walls was monitored with a fluorescence microscope (Axiovert with FITC filter; Carl Zeiss Meditec, Oberkochen, Germany; with Improvision Openlab software; Coventry, UK). The total number of leukocytes adhering in the retinal vasculature was counted and compared between AGE-Alb–treated mice and Alb-treated mice.

Statistical Analysis

All results were expressed as the mean $\pm$ SEM. Paired groups of two with equal variance were compared using a two-sample t-test. Differences were deemed statistically significant when $P < 0.05$.

RESULTS

Analysis of the CML and CEL Content of AGE-Alb and Native Alb

The CML and CEL content of AGE-Alb and native unmodified Alb were analyzed by GC-MS. The lysine content of the samples was analyzed by cation-exchange chromatography and values were expressed as millimoles CML or CEL per mole lysine (Table 1).

The AGE-Alb used in this study contained 23.85 mmol CML/mol lysine compared with a control value of 0.286 mmol CML/mol lysine. These results demonstrate a significant modification of the protein by the reactive intermediate glycoaldehyde, resulting in the generation of at least one major quantifiable end product, namely CML. The low level of CEL formed served as a control for the reaction, and the increase noted in AGE-Alb compared with that of native albumin was not statistically significant. In addition, BSA modified to specifically yield

TABLE 1. Analysis of the CML and CEL Content of AGE-Alb and Native Alb Using GC-MS and Cation-Exchange Chromatography

Albumin Sample	CML (mmol/mol lysine)	CEL (mmol/mol lysine)
Control albumin (Alb)	0.286	0.09
Glycoaldehyde-modified albumin (AGE-Alb)	23.85	1.05

The AGE-Alb used in the study contained 23.85 mmol CML/mol lysine compared with a control value of 0.286 mmol CML/mol lysine. These results demonstrate a significant modification of the protein by the reactive intermediate glycoaldehyde, resulting in the generation of at least one major quantifiable end product, CML.

CML was prepared and characterized. The specificity and degree of modification was quantified by GC-MS analysis (Table 2).

The present study showed that unmodified, native albumin contains 0.146 mmol/mol lysine CML, whereas mildly (10%) and highly (35%) modified albumin contains 32.6 and 88.5 mmol/mol lysine CML, respectively. The fact that the CEL was not increased demonstrates the specificity of the reaction for CML formation.

In Vitro Experiments

Activation of NFκB by AGE-Alb. As determined by EMSA, AGE-Alb caused a marked increase in NFκB DNA binding when compared with native, control albumin (Fig. 1A, AGE-Alb versus Alb, lane 3 versus lane 4). Quantification of p65 nuclear protein by ELISA revealed a statistically significant increase in the amount of p65 protein in the nuclei of AGE-Alb–treated RMECs compared with Alb-treated control cells (Fig. 1B, AGE-Alb versus Alb, optical density [OD] 2.3 vs. 2.0, $n = 3$, $P = 0.0045$).

Increased Leukocyte Adhesion to Endothelial Cells Treated with AGE-Alb. An in vitro cell adhesion assay was performed on endothelial cells treated with AGE-Alb or native Alb (100 μg/mL for 4 hours). Isolated leukocytes consistently displayed increased adhesion to endothelial cell monolayers treated with AGE-Alb. Adherence levels were similar to the adhesion seen with the positive control TNFα. Quantification of the number of leukocytes adhering to AGE-treated endothelial cells (AGE-Alb) revealed a statistically significant increase when compared with the control (Alb; Fig. 2). Results represent the average of 12 experiments ± SEM (AGE-Alb–treated versus Alb control, 202% ± 21% vs. 100% ± 10%, $P < 0.01$).

Effect of Co-incubation of sRAGE on AGE-Induced Leukocyte Adhesion. An in vitro cell adhesion assay was performed on endothelial cells treated with co-incubation of sRAGE+AGE-Alb or sRAGE+native Alb for 4 hours. Treatment of cells using co-incubation of AGE-Alb with sRAGE (1500

TABLE 2. Analysis of the CML Content in CML-Modified Bovine Serum Albumin

Albumin Sample	CML (mmol/mol lysine)	CEL (mmol/mol lysine)
Control BSA	0.146	0.109
10% CML-BSA	32.6	0.091
35% CML-BSA	88.5	0.101

Unmodified/native albumin contained 0.146 mmol/mol lysine CML while mildly (10%) and highly (35%) modified albumin contained 32.6 and 88.5 mmol/mol lysine CML, respectively. The fact that the CEL was not increased demonstrated the specificity of the reaction for CML formation.
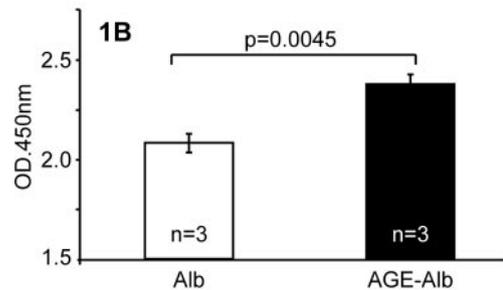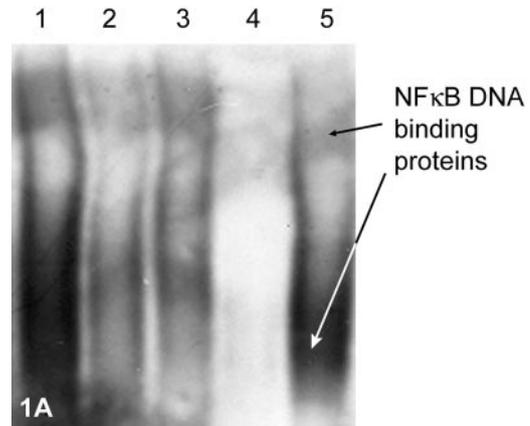

FIGURE 1. **(A)** EMSA for detection of NFκB DNA-binding proteins. Retinal endothelial cells were treated with (*lane 1*) TNFα, (*lane 2*) hydrogen peroxide, (*lane 3*) AGE-Alb (150 μg/mL), (*lane 4*) Alb (150 μg/mL), and (*lane 5*) AGE-BSA for 2 hours. Nuclear extraction was performed followed by EMSA for detection of DNA binding NFκB proteins. In AGE-Alb–treated endothelial cells, increased NFκB DNA binding was noted (*lane 3*). No binding was noted in control cells treated with native Alb (*lane 4*). **(B)** Quantification of nuclear NFκB in AGE-Alb–treated RMECs. NFκB p65 protein was quantified in nuclear extracts from RMECs treated for 2 hours with AGE-Alb of native Alb (150 μg/mL) using an ELISA. A statistically significant increase in p65 was noted in those cells treated with AGE-Alb compared with those treated with native Alb. Results represent the average of three experiments ± SEM (AGE-Alb versus Alb OD, 2.3 vs. 2.0, $P = 0.0045$).

μg/mL) caused a significant reduction in the amount of AGE-induced leukocyte adhesion to endothelial cell monolayers (Fig. 2). Control experiments performed using the same concentration of nonimmunized mouse IgG (Sigma-Aldrich) to test for the effect of co-incubation with a generic protein showed no effect on AGE-induced adhesion (results not shown). Results represent the average of 12 experiments ± SEM (AGE-Alb+sRAGE vs. AGE-Alb, 114% ± 13% vs. 202% ± 21%, $P < 0.01$).

In Vivo Experiments

To investigate the effect of AGEs on retinal vascular cells, further experiments were performed in vivo by treating mice with murine AGE-Alb or the Alb control every day for seven consecutive days.

Effect of AGE-Alb on Retinal ICAM-1 mRNA Copy Number. A statistically significant increase was detected in retinal ICAM-1 mRNA expression using a quantitative real time PCR analysis. An almost twofold increase was noted in AGE-treated retinas when compared with native Alb control retinas as early as 48 hours after the first infusion. The increase was still evident 7 days later (1.8-fold increase from control, $P < 0.001$, $n = 20$, Fig. 3A). In addition, immunohistochemical analysis of
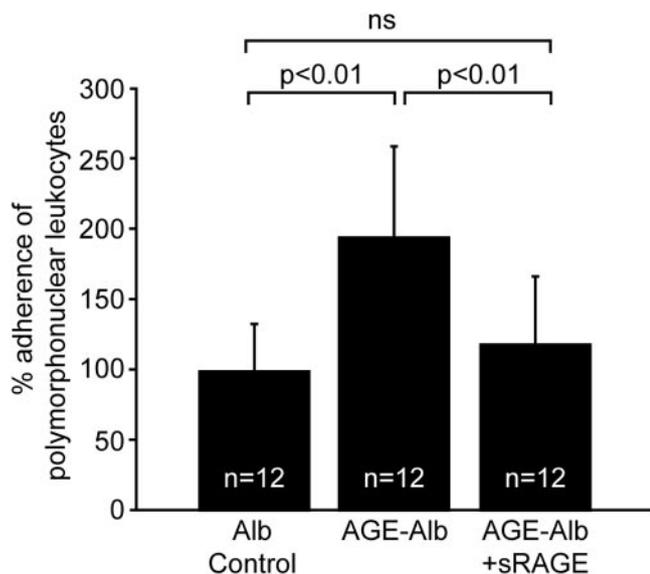
FIGURE 2. In vitro leukocyte cell adhesion assay. Quantification of the number of adhering leukocytes. A statistically significant increase was found in the number of leukocytes adhering to AGE-Alb–treated cells (100 μg/mL for 4 hours) compared with the number that adhered to control cells treated with native albumin. Results represent the average of 12 experiments $\pm$ SEM (AGE-Alb treated versus Alb control, 202% $\pm$ 21% vs. 100% $\pm$ 10%, $P < 0.01$). Effect of co-incubation of sRAGE on AGE induced leukocyte adhesion. Treatment of cell with co-incubation of AGE-Alb with sRAGE (1500 μg/mL for 4 hours) caused a significant reduction in the amount of AGE-induced leukocyte adhesion to endothelial cell monolayers. Control experiments performed using the same concentration of nonimmunized mouse IgG (Sigma-Aldrich) to test for the effect of co-incubation with a generic protein showed no effect on AGE-induced adhesion (results not shown). Results represent the average of 12 experiments $\pm$ SEM (AGE-Alb+sRAGE versus AGE-Alb, 114% $\pm$ 13% vs. 202% $\pm$ 21%, $P < 0.01$).

retinas from mice infused with AGE (10 mg/kg, 7 days) demonstrated increased ICAM-1 protein expression in the retinal vessels (Fig. 3C) when compared with Alb-infused control mice (Fig. 3B).

Effect of AGE-Alb on Retinal NFκB Expression. When activation of retinal NFκB was analyzed, a significant increase in DNA binding NFκB protein was detected in retinas of mice treated with AGE-Alb compared with those treated with native albumin (AGE-Alb versus Alb, 0.23 vs. 0.076, $P < 0.0001$, $n = 10$, Fig. 4).

In Vivo Leukostasis Assay. An in vivo staining method was used to determine whether more leukocytes adhered to the retinal vasculature of the AGE-treated animals. In comparison to control mice, AGE-Alb–infused animals showed a statistically significant increase in the number of leukocytes visible in the retinal capillary bed after removal of nonadherent cells by perfusion.

A direct quantification of the number of leukocytes was performed in all retinas and results represent the average $\pm$ SEM (Fig. 5, AGE-Alb versus Alb, 6.89 $\pm$ 0.58 vs. 2.53 $\pm$ 0.31 $n = 12$, $P < 0.05$). The micrographs are representative of stationary leukocytes counted in the retinal vasculature of AGE-Alb–treated mice.

AGE-Alb and Inner Blood–Retinal Barrier Breakdown. Mice treated with AGE-Alb demonstrated a fivefold increase in leakage of Evans blue dye, demonstrating a breakdown of the blood–retinal barrier (AGE-Alb versus control Alb, 8.2 μL vs. 1.6 μL plasma $\times$ grams retina dry weight $\times$ hours, $P < 0.001$, Fig. 6).
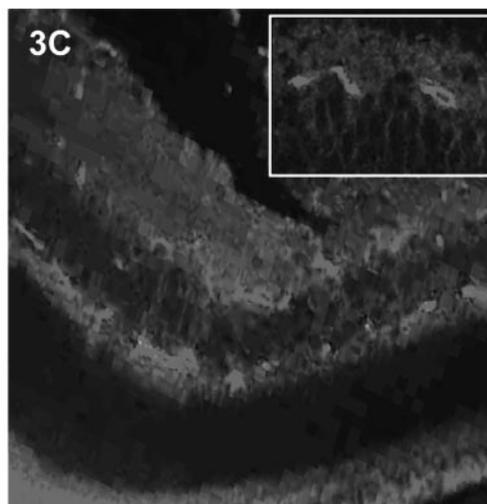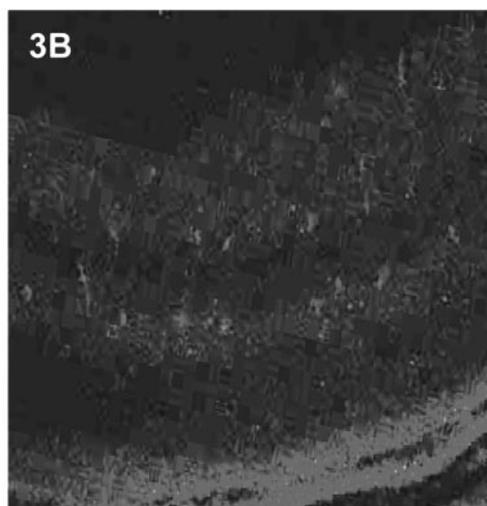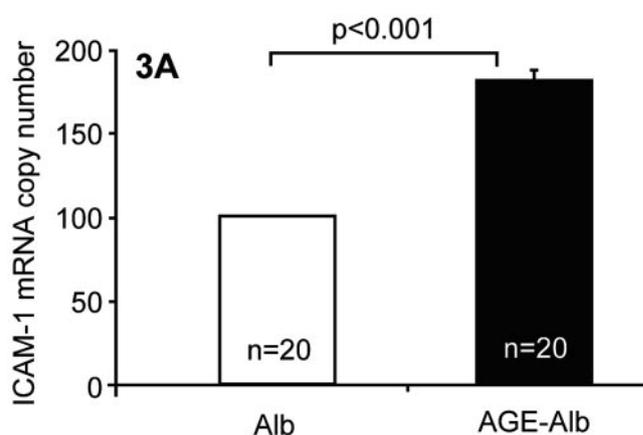

FIGURE 3. (A) Effect of AGE-Alb infusion on mouse retinal ICAM-1 mRNA expression. A statistically significant increase in retinal ICAM-1 mRNA expression was detected. An almost twofold increase was noted in AGE-Alb–treated mice when compared with Alb control retinas as early as 48 hours after the first infusion, which remained after 7 days. Results represent the average of 20 experiments $\pm$ SEM (1.805-fold increase over control, $P < 0.001$). (**B, C**) Immunohistochemical analysis of ICAM-1 protein expression on retinas from AGE-infused mice. Mice received native albumin (**B**) or glycated albumin (**C**) intraperitoneally (IP) every day for 10 days (10 mg/kg). Retinas were removed, flatmounted, and stained for ICAM-1. Increased intensity and more frequent staining were observed in those retinas removed from AGE-Alb infused mice (**B**).
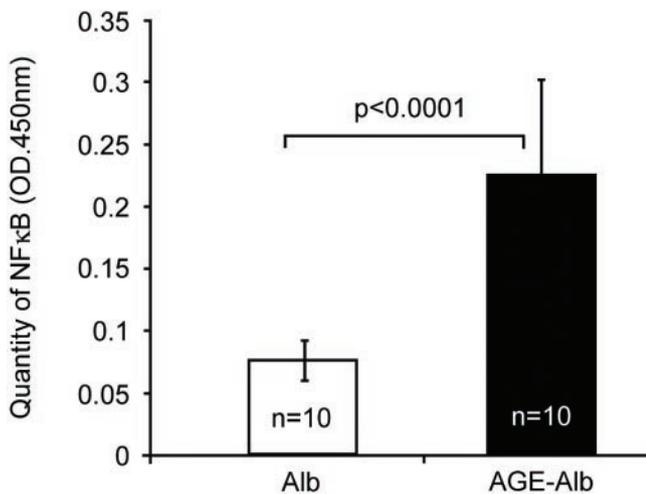

FIGURE 4. Effect of AGE-Alb infusion on mouse retinal NFκB. Mice were injected (IP) with AGE-Alb (10 mg/kg) or native Alb every day for seven consecutive days. Eyes were enucleated and retinas removed for nuclear extraction followed by measurement of NFκB. There was a statistically significant increase in the amount of retinal NFκB DNA binding proteins noted in mice treated with AGE compared with those infused with native Alb (AGE-Alb versus Alb, 0.23 vs. 0.076, $P < 0.0001$, $n = 10$ mice).

DISCUSSION

The inappropriate adherence of leukocytes to the retinal capillary endothelium has been established as an important pathogenic event in animal models of diabetic retinopathy and plays a significant role in the breakdown of the blood–retinal barrier and retinal capillary occlusion.[17] Leukostasis is a manifestation of chronic inflammation in the retinal microvasculature and is dependent on a complex interplay between distinct cell types. The phenomenon appears to be modulated by diabetes-related activation of circulating leukocytes coupled with the marked upregulation of adhesion molecules such as ICAM-1 by the microvascular endothelium.[18,30] In the present study, we adopted several investigative approaches to establish a role for advanced glycation in retinal leukostasis. It was important to consider activation of the ICAM-1 gene, which is controlled by transcription factors (such as NF-κB) that are closely linked to AGE-mediated generation of oxidative stress.[31] We hypothesized that the adhesion of leukocytes, both in vitro and in vivo, was a functional representation of AGE-mediated dysfunction of the retinal vascular endothelium.

AGEs are known to circulate at high levels in diabetic persons,[32] and the model adopted in the present study helps to dissect some of the complexities of the diabetic milieu. Previous studies have demonstrated that AGEs infused into normoglycemic animals accumulate in the retinal microvasculature and can induce diabetic-like retinal vascular lesions, such as loss of capillaries, blood–retinal barrier dysfunction, and VEGF upregulation.[11,33] In the kidney, long-term AGE infusion can induce diabetes-like glomerulosclerosis in nondiabetic animals.[34,35] Such approaches have limitations, although they can present a model in which acute effects of circulating AGEs can be studied within an in vivo system.

Although the AGE-albumin used in the present study represents a model AGE, glycoaldehyde modification is physiologically relevant, because this aldehyde is known to occur at elevated levels in diabetic individuals and serves as an important intermediate for AGE formation in vivo.[36] Furthermore, albumin modified by glycoaldehyde has been shown to act as a functional ligand for the class-A macrophage scavenger receptor (MSR-A) with the likelihood of interacting with other

known AGE-receptors. In the present study, we have shown that glycoaldehyde modification of albumin can lead to appreciable levels of CML, and this compares well with a report by Nagai et al.[22] CML itself can lead to oxidative stress in endothelial cells,[37] and it has been suggested that this AGE can directly stimulate the receptor for AGE (RAGE) thus activating key cell-signaling pathways, such as NF-κB, and modulating gene expression.[38,39] The involvement of RAGE signaling pathways in retinal leukostasis remains an important area for study.

Accumulation of AGEs, both within vessel walls and as complex modifications of serum proteins, has been shown to induce proinflammatory responses.[33,40] These adducts can activate leukocytes[41,42] and promote upregulation of the adhesion molecules VCAM-1 and ICAM-1 on the surface of macrovascular endothelial cells, phenomena that are central to the role of AGEs in atherogenesis.[33,43,44] Despite their increasingly recognized role in macrovasculopathy, AGEs have received little attention in diabetes-related inflammation in the retina. The current investigation has demonstrated that advanced gly-
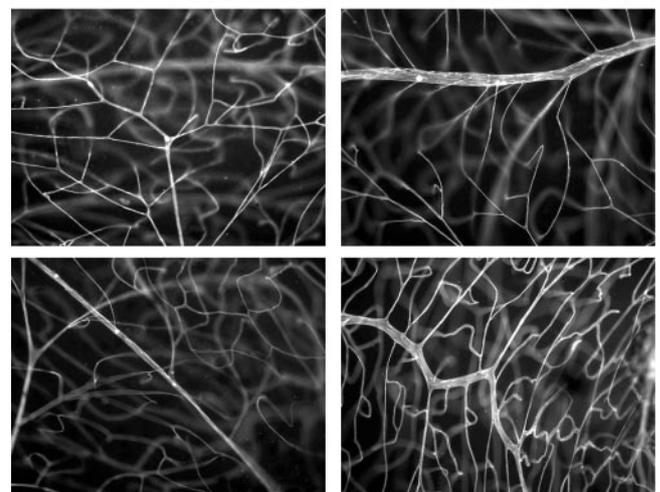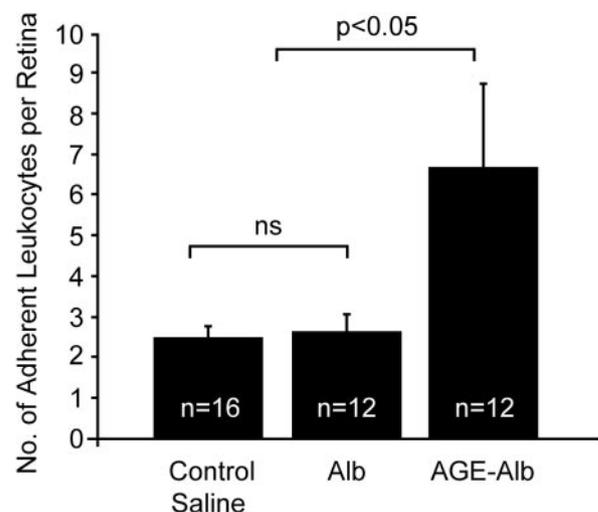

FIGURE 5. Effect of AGE-Alb infusion on mouse retinal leukostasis. Mice were injected (IP) with AGE-Alb (10 mg/kg) or native Alb every day for seven consecutive days. The number of leukocytes per retina was counted after perfusion and increased leukostasis was noted in the retinas of treated mice compared with Alb-treated control retinas. Data represent the average ± SEM of at least 12 experiments (AGE-Alb versus Alb, 6.89 ± 0.58 vs. 2.53 ± 0.31, $P < 0.05$). Micrographs are representative of stationary leukocytes counted in the retinal vasculature of AGE-Alb–treated mice.
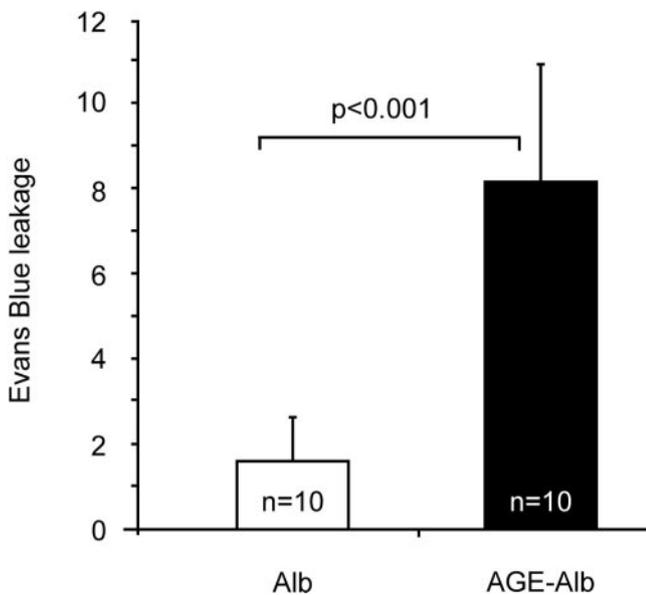
FIGURE 6. Effect of AGE-Alb infusion on the blood–retinal barrier. Retinal permeation by Evans blue dye was assessed in mice infused with native Alb or glycated Alb. A statistically significant increase in the amount of dye leakage from the retinal vasculature was noted in AGE-treated mice, indicative of breakdown of the blood–retinal barrier in AGE-infused mice (AGE Alb versus Alb, 8.2 vs. 1.6 μL plasma $\times$ grams retina dry weight $\times$ hours, $P < 0.001$).

cation may play a significant role in retinal microvascular occlusion by promoting endothelial cell responses that enhance leukocyte adhesion to capillaries.

The ability of AGE-Alb to induce substantial activation of ICAM-1 in the retinal microvascular endothelium, possibly through increased transcription of NFκB, is fresh evidence that these adducts may play a hitherto unrecognized role in retinal leukostasis. It has been demonstrated previously that ICAM-1 mRNA or protein levels need not be upregulated much beyond 1.5-fold to initiate marked adherence of leukocytes to the retinal capillaries.[18] In the present study, a small, yet significant, elevation in retinal ICAM-1 and NFκB activation occurred very soon after AGE-Alb infusion (48 hours), and levels were maintained at a consistently high level throughout the period of treatment. Retinal leukostasis and blood–retinal barrier dysfunction occurred after the infusion of AGE into normal mice, and these phenomena may be closely related to the aforementioned endothelial effects.

Blood–retinal barrier dysfunction is an established lesion of diabetic retinopathy. The basis of abnormal retinal microvascular leakage remains equivocal, although it has been shown that VEGF plays a key vasopermeability role[29,45] probably modulated through ICAM-1 and leukocyte adherence to the endothelium.[46] The current investigation has confirmed previous reports that infused AGEs can lead to blood–retinal barrier breakdown in vivo, and it is significant that this seems to correlate spatially with ICAM-1 upregulation and leukostasis. Effective neutralization of ICAM-1 can prevent blood–retinal barrier breakdown in diabetic animals.[18] Further research is needed to establish whether AGE-mediated blood–retinal barrier dysfunction is modulated by blockage of ICAM-1.

Acknowledgments

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