Receptor for Advanced Glycation End Products and Age-Related Macular Degeneration

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Purpose. Advanced glycation end products (AGE) exacerbate disease progression through two general mechanisms: modifying molecules and forming nondegradable aggregates, thus impairing normal cellular/tissue functions, and altering cellular function directly through receptor-mediated activation. In the present study receptor for AGE (RAGE)–mediated cellular activation was evaluated in the etiology of human retinal aging and disease.

Methods. The maculas of human donor retinas from normal eyes and eyes with early age-related macular degeneration (AMD) and advanced AMD with geographic atrophy (GA) were assayed for AGE and RAGE by immunocytochemistry. Cultured ARPE-19 cells were challenged with known ligands for RAGE, AGE, and S100B, to test for activation capacity. Immunocytochemistry, real-time RT-PCR, immunoblot analysis, and the TUNEL assay were used to determine the consequences of RPE cellular activation.

Results. Little to no immunolabeling for AGE or RAGE was found in photoreceptor and RPE cell layers in normal retinas. However, when small drusen were present, AGE and RAGE were identified in the RPE or both the RPE and photoreceptors. In early AMD and GA, the RPE and remnant photoreceptor cells showed intense AGE and RAGE immunolabeling. Both AGE and S100B activated cultured RPE cells, as revealed by upregulated expression of RAGE, NFκB nuclear translocation, and apoptotic cell death.

Conclusions. Immunolocalization of RAGE in RPE and photoreceptors coincided with AGE deposits and macular disease in aged, early AMD, and GA retinas. Further, AGE stimulated RAGE-mediated activation of cultured ARPE-19 cells in a dose-dependent fashion. AGE accumulation, as occurs with normal aging and in disease, may induce receptor-mediated activation of RPE/photoreceptor cells, contributing to disease progression in the aging human retinas. (*Invest Ophthalmol Vis Sci.* 2004;45:3713–3720) DOI:10.1167/iovs.04-0404

Although genetic and environmental factors contribute to age-related macular degeneration (AMD), specific mechanisms of disease onset and progression remain unclear. Accumulated macular drusen, extracellular deposits in Bruch's membrane, are a risk factor for AMD.[1,2] Retinal pigment epithelium (RPE) dysfunction may decrease photoreceptor cell viability, leading to central vision loss in AMD.[3] Studies have shown degenerative cellular and molecular perturbations in RPE and photoreceptors overlying macular (and extramacular) drusen.[3–5]

In environments of oxidant stress, as in aging tissues, formation and accumulation of advanced glycation end products (AGE) are accelerated (reviewed in Ref. 6). Extracellular and cellular proteins are modified by the Maillard reaction,[7,8] nonenzymatic reactions of aldehyde groups of sugars and ϵ-amino groups in proteins, forming Schiff bases and Amadori adducts. Subsequent rearrangement reactions form stable, irreversibly bound AGE. The auto-oxidation of polyunsaturated fatty acids (PUFAs) can also contribute to carboxymethyllysine (CML) AGE formation.[9,10] CML-AGE is a known marker for oxidatively stressed tissues in many diseases. Cross-linking of long-lived extracellular matrix proteins and the inability of cells to degrade AGE intracellularly ultimately impair cellular physiology.

Disease progression by the formation of AGE is further mediated by chronic cellular activation induced by the AGE receptor (RAGE).[11] RAGE is typically upregulated in response to injury or inflammation, effecting localized tissue repair and reinstating homeostasis. RAGE, a member of the immunoglobulin superfamily of cell surface molecules, interacts with several ligands, including AGE, amyloids, S100/calgranulins, and amphoterin. Enhanced formation and accumulation of RAGE ligands, as occurs in aging and chronic disorders, provides a trigger for prolonged cellular activation by RAGE. Sustained RAGE-mediated cellular activation has been shown to contribute to disease progression in diabetes, Alzheimer's disease, rheumatoid arthritis, elastosis, pulmonary fibrosis, and various cancers.[12–17] AGE and other RAGE ligands activate $p21^{ras}$, MAP ERK1/2 kinases, and NFκB nuclear translocation, altering expression of genes involved with cellular stress.[17] RAGE expression is upregulated by this pathway, initiating a positive feedback loop sustained by continued ligand availability. Elucidation of RAGE-mediated alterations in gene expression of adhesion molecules, cytoskeletal and matrix proteins, inflammatory cytokines, and apoptosis mediators has been instrumental in linking mechanisms of cellular activation to disease progression.

AGE have been reported to accumulate in aging and AMD eyes in Bruch's membrane, drusen, subfoveal neovascular membranes, and RPE cells.[18,19] However, in previous studies, specific RAGE-mediated RPE cellular response to these adjacent regions of AGE deposition was not addressed. Of note, Hammes et al.[20] showed CML-AGE, RAGE cellular-activating

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events (including NEκB nuclear localization) in neovascular membranes excised from patients with wet AMD. However, in that report, the excised subretinal membranes were highly fibrotic and did not include an identifiable neuroretina. Furthermore, the RAGE-positive cells were identified as infiltrating macrophages. Comparative RAGE labeling in RPE and photoreceptor layers from earlier stages, normal aged and dry AMD retinas, was not examined.

Based on evidence of RPE dysfunction, oxidative stress, and adjacent regions of AGE accumulation in aging and AMD eyes, AGE/RAGE-mediated RPE cellular activation may play a role in disease progression. Therefore, earlier stages of aged and AMD macular sections were examined for endogenous RPE AGE/RAGE expression before end-stage AMD and RPE cells were tested in culture for RAGE-mediated cell responsiveness.

Materials and Methods

Immunocytochemistry

Cryostat sections (10–12 μm thick) of maculas of two donors with early AMD, three with GA, and one with drusen and seven aged normal donor eyes were obtained from the FFB Eye Donor Program. The study was conducted in accordance with the guidelines in the Declaration of Helsinki for research involving human tissue. Macular tissue sections from donors with early AMD and from aged control and drusen-containing eyes were identified by histopathology, clinical records, or both. Antibodies to AGE (Clone No. 6D12; Wako Bioproducts, Richmond, VA), RAGE N42-59 (Research Diagnostics Inc., Flanders, NJ), and phospho-IκB-α (Cell Signaling Technology, Beverly, MA), were used according to manufacturers' suggested concentrations. Because of the inherent autofluorescence of lipofuscin in aging RPE and drusen in AMD eyes, streptavidin biotin peroxidase immunocytochemistry was performed (Universal Quick Kit and AEC kit; both from Vector, Burlingame, CA). Preimmune serum (10%) or preabsorption of the antibody with corresponding peptides (20 μg/mL) were used to confirm antibody specificity (Fig. 1J, inset) RDI (N42-59) hRAGE, and 6D12 AGE antibodies were used for immunocytochemical detection of RAGE and AGE, respectively. 6D12 has been used to study AGE formation in aging and AMD eyes.[18,20] Because a limited number of sections from donor eyes were available, not all specimens could be assayed for AGE. Seven of 13 retinas, including age-matched normal, early AMD, and GA retinas were assayed for both AGE and RAGE.

ARPE-19 Cell Culture

ARPE-19 cells were grown in 10% fetal bovine serum (FCS)/RPMI1640/gentamicin (Invitrogen-Gibco, Carlsbad, CA). Cells were seeded in six-well plates coated with a 1:16 dilution of low growth factor synthetic basement membrane (Matrigel; BD Biosciences, Bedford, MD) in RPMI1640 medium. On confluence, the cells were incubated in 1% fetal calf serum (FCS)/RPMI1640/gentamicin for at least 2 to 3 weeks before treatments, to allow for differentiation. AGE-bovine serum albumin (BSA), BSA, or S100B (Sigma-Aldrich, St. Louis, MO) was added to the medium at 0-, 2-, 5-, 10-, and 25-μM concentrations for dose-response experiments. Cells were treated for 1 to 6 days. Control wells were treated with a 1:1000 dilution of a RAGE N-42-59 (Research Diagnostics Inc.) antibody, which was generated against amino acids 42-59 located in the extracellular ligand binding domain of the receptor. On completion of the experiments, cells were fixed and processed for immunocytochemistry and TUNEL assays.

AGE-BSA Preparation

Fatty acid–free BSA (5.0 mg/mL; Sigma-Aldrich) was incubated with 33 mM glycolaldehyde (Sigma-Aldrich) in PBS for 5 days at 37°C, according to the protocol of Nagai et al.[21] Glycolaldehyde modification of BSA has been shown reproducibly to bind RAGE with high affinity.[22] The AGE-BSA product was dialyzed in PBS for 4 days to remove unbound glycolaldehyde before cell treatments. AGE modification was confirmed with Western blot using the 6D12 antibody (Wako Bioproducts) recognizing AGE-modified products, including CML-AGE. The BSA control sample showed no labeling with the AGE antibody.

Real-Time RT-PCR

A commercial system (Opticon; MJ Research, Watertown, MA) was used for quantifying RAGE transcript levels in ARPE-19 cell experiments. Total RNA was then extracted (RNeasy; Qiagen, Valencia, CA). RT-PCR was performed QuantiTect SYBR Green RT-PCR; Qiagen) on 150 ng total RNA from each sample, followed by PCR amplification of specific RAGE transcripts. The RAGE primers were designed to identify only the full-length product using a 5′ primer straddling splice junctions for exons 1 and 2 and 3′ primer exons 3 and 4 (RT-RAGEf 5′-GCT GGA ATG GAA ACT GAA CAC AGG-3′; RT-RAGEr 5′-TTC CCA GGA ATC TGG TAG ACA CG-3′). Standard curves were generated from 0 to 500 ng total RNA from untreated groups. 18S internal standards (3:7 primer to competitor; QuantumRNA; Ambion, Austin, TX) were amplified in parallel samples for normalizing the RAGE signal to equivalent 18S signal. Results are represented as RAGE levels (arbitrary fluorescence units) relative to that of the BSA control. The resultant graph represents data from three separate experiments, duplicate wells assayed in triplicate.

TUNEL Assay

Cells were assayed for apoptosis (APO-BRDU-IHC kit; Phoenix Flow Systems, San Diego, CA). TUNEL-positive cells are reported as a percentage of total cell counts per well. At magnification 250×, total and apoptotic cells were counted from five random visual fields for each four- to six-well treatment in two separate experiments.

Western Blot

In parallel experiments, cells were removed from synthetic matrix (Matrigel; BD Biosciences) with dispase (2.4 U/mL; Sigma-Aldrich) and sonicated in lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA; and 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride [PMSF], and 1× protease inhibitor cocktail; Roche Diagnostics, Indianapolis, IN). Protein concentrations were determined with the Bradford assay. Fifteen micrograms protein from each sample was separated on a 12% SDS-PAGE, transferred onto nitrocellulose filters, and probed with 1:100 dilution anti-RAGE (Santa Cruz Biotechnology, Santa Cruz, CA) or 1:1000 dilution anti-RAGE (Chemicon, Temecula, CA). These antibodies allow for specific detection of full-length RAGE, unlike the N42-59 N-terminal RDI RAGE antibody that detects secretory RAGE in addition to full-length RAGE. Results are representative blots from triplicate wells for three separate experiments.

Results

AGE and RAGE in Aged Normal Retinas

As observed by Hammes et al.,[20] RAGE immunolabeling was present in the plexiform and ganglion cell layers, as well as cells in the inner nuclear layer. RAGE labeling in the inner retina or choroid ranged from slight to intense labeling in normal aged maculas. We found that regional variation in AGE and RAGE immunolabeling within the sections provided an internal control for our study. For example, a normal donor retina (82 years old) showed, within a region containing no obvious disease, some AGE labeling in the RPE, but little reactivity within the photoreceptor layer (Fig. 1A). Adjacent regions with drusen, had additional labeling especially evident within the inner segments (IS) of photoreceptors (Fig. 1B). Sections with no disease had little to no RAGE labeling in the RPE or photoreceptors (Figs. 1C, 1E). Adjacent regions with AGE immunoreactivity had enhanced RAGE signals in the RPE and photoreceptor IS (Figs. 1D, 1E). Labeling for both AGE and

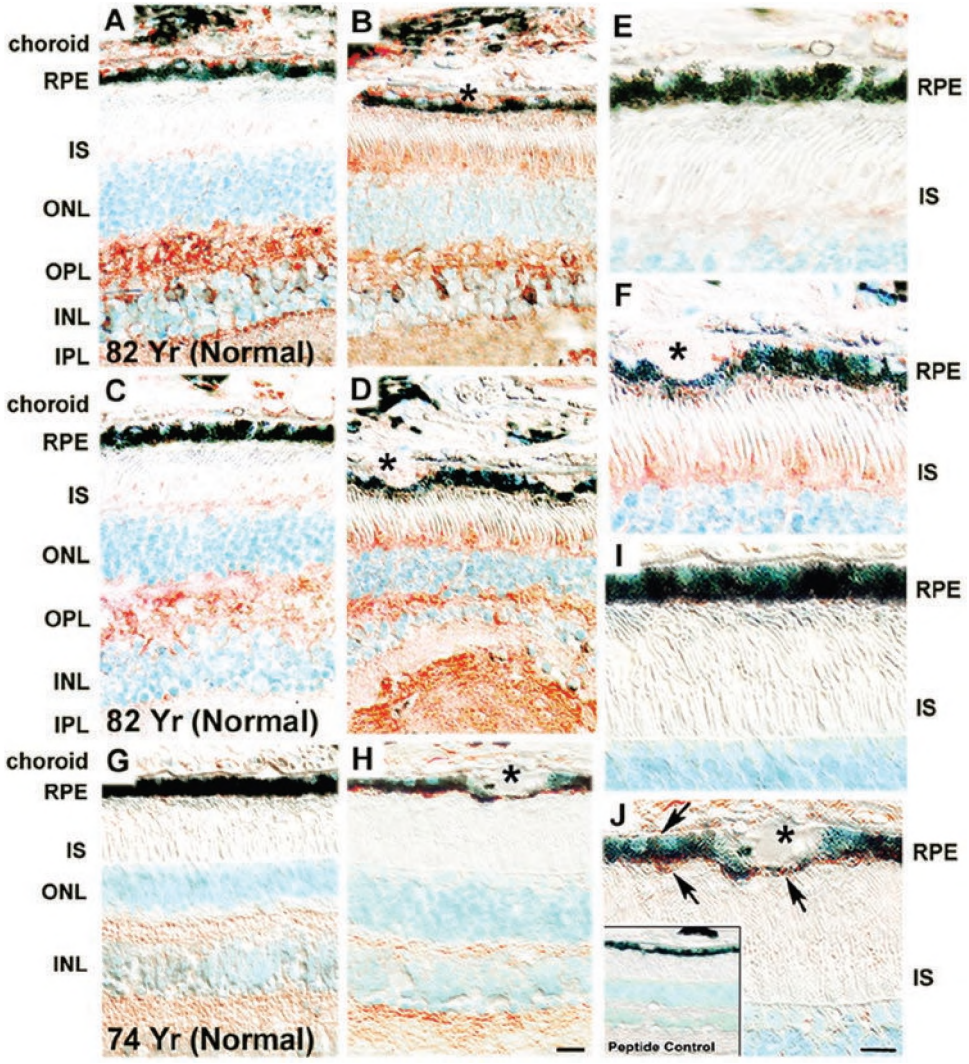

FIGURE 1. Immunocytochemistry for AGE and RAGE in macular sections from age-matched retinas. Immunopositive signal is *red*. (**A**, **B**) AGE ICC and (**C**, **D**) RAGE ICC in similar regions from an 82-year-old age-matched normal donor retina. (**A**) AGE labeling was apparent between Bruch's membrane and the RPE but absent in photoreceptors. (**B**) In an adjacent region with drusen (✱), AGE-immunopositive labeling was also present in the photoreceptor IS region. (**C**, **D**) Corresponding regions immunolabeled for RAGE. In (**C**), the RPE and photoreceptors showed little RAGE immunolabeling, but in the adjacent region (**D**) with drusen (✱), there was RAGE immunolabeling in RPE and the IS of the photoreceptors. (**E**, **F**) Higher magnification of RAGE labeling in RPE and photoreceptors from (**C**) and (**D**). (**G**–**J**) RAGE labeling for another age-matched control retina (74 years old). (**G**) Sparse, diffuse labeling for RAGE in the RPE and no labeling were shown in the photoreceptor IS or ONL. (**H**) In the same macular section, there was RAGE label in the RPE over drusen (✱). (**I**, **J**) Higher magnifications from (**G**) and (**H**). (**J**, *inset*) A 74-year-old macular section showed no immunolabeling when antibody was preabsorbed with peptide (20 μg/mL). Cell nuclei were stained with methyl green. Scale bars, 25 μm.

RAGE was found on both apical and basal sides of RPE cells but was also primarily associated with the photoreceptor IS. The outer segment and outer nuclear layers had little reactivity for either AGE or RAGE in sections from this retina. AGE labeling was particularly heavy in the cytoplasm of unidentified cells in the INL but there was no RAGE labeling in corresponding sections. The OPL was relatively positive for both AGE and RAGE in all retinal regions. In comparison, RAGE immunolabeling was very low throughout the retina of a normal 74-year-old donor, with light labeling in cells of the choroid and plexiform layers and occasional labeling in INL cells. However, RPE cells showed enhanced RAGE signal in an adjacent region with drusen but no obvious label in photoreceptor cells (Eigs. 1G–J). Results if RAGE immunocytochemistry (ICC) on normal eyes are summarized in Table 1.

AGE Products and RAGE in GA and AMD Retinas

AGE immunolabel was evident throughout the sections of GA maculas. One GA macula (76 years old) showed a thickened Bruch's membrane and large drusen with AGE on basal and apical sides of RPE cells. In addition, a region of this macula with a relatively intact photoreceptor cell layer showed AGE labeling in the IS region and some localization around outer nuclear layer (ONL) nuclei. RAGE signals were essentially identical with those of AGE within this region (Eigs. 2A–D). Intense labeling was also evident for both AGE and RAGE in the choroid, possibly reflecting monocyte/macrophage infiltration due to a region of choroidal neovascularization (not shown) reported in this donor. An older donor with GA (85 years old) had intense AGE labeling in the atrophied photoreceptor and the RPE cell layers (Eig. 2E). A corresponding region showed an identical profile of RAGE labeling in the RPE and throughout the photoreceptors cell layers, including the outer segments (OS), IS, and ONL (Figs. 2F–H). AGE labeling was intense in Bruch's membrane overlying large drusen. The choroid had weak AGE and RAGE labeling. AGE-positive cells in the INL also had weak RAGE labeling.

Retinas of eyes with early AMD showed AGE/RAGE profiles similar to those in GA donor eyes. AGE (Eig. 2I) and RAGE (Eigs. 2J–L) labeling was correspondingly found in RPE and photoreceptor IS and ONL (when present). In both AMD eyes, choroidal cells showed intense labeling for both AGE and RAGE. Results for RPE and photoreceptor labeling in GA and AMD eyes are summarized in Table 1.

ARPE-19 Cell Activation

Cultured ARPE-19 cells were treated with AGE-BSA and equimolar concentrations of BSA control. AGE-BSA was made as previously described.[21] A Western blot using the 6D12 antibody demonstrated AGE- BSA product generation as opposed to the unmodified BSA control (Eig. 3A).

TABLE 1. Summary of RPE and Photoreceptor AGE and RAGE Immunolabeling in Normal Aged, Early AMD, and GA Retinas

Accession #	Age	Case	Drusen	RPE RAGE	Photoreceptor RAGE
99-11-23	58	Normal	N	−	−
0012A151	73	Normal	N	−/+	−/+
6-23-00	74	Normal	Y	+	−
00-0164	75	Normal	Y	+	+
00-46	78	Normal	Y	+	+
95-10	79	Normal	Y	+	+
				AGE/RAGE	AGE/RAGE
1-41	82	Normal	Y	+/+	+/+
99-35	76	Drusen	Y	+/+	+/+
00-48	76	GA	Y	+/+	+/+
99-30	85	GA	Y	+/+	+/+
00-11	94	GA	Y	+/+	+/+
FFB#678	95	AMD (dry)	Y	+/+	+/+
FFB#577	70	AMD (dry)	Y	+/+	NA

$n = 13$.

Twenty-five-micromolar AGE concentrations induced total cell death within 24 to 48 hours. Data shown are results from ARPE-19 cells exposed to 2 to 10 μM AGE concentrations, collected after 4 days' exposure to respective treatments. To show that AGE induce receptor-mediated cellular activation, ARPE-19 cells were assayed for downstream effects known to be mediated by RAGE.[15–17] To examine this interaction in ARPE-19 cells, phosphorylated IκB-α was used as a marker for NFκB nuclear translocation. IκB-α is an inhibitory molecule that binds and sequesters the transcription factor NFκB to the cytoplasm when unphosphorylated. On phosphorylation, IκB-α releases NFκB for nuclear translocation and subsequent gene transcriptional regulation.[23] Treatments with micromolar AGE-BSA revealed strong immunopositive signal for the phosphorylated form of IκB-α, unlike the BSA control-treated cells (Fig. 3B). These results indicate that exposure to AGE induces NFκB nuclear activation in RPE cells.

Induction of Apoptosis in ARPE-19 Cells

ARPE-19 cells were examined for RAGE-ligand–induced apoptosis. Both RAGE ligands, AGE and S100B, were used as RAGE-specific ligands in the treatments. S100B is a central nervous system (CNS) neurotrophic factor at normal concentrations but is reported to be neurotoxic at higher concentrations. Treated ARPE-19 cells showed altered morphology and regions of cell clumping in contrast to the more differentiated phenotype (Fig. 3C). Micromolar concentrations of AGE-BSA and S100B induced apoptosis (Fig. 3C, arrows). Results in Figure 3D summarize TUNEL-positive reactions after 4 days of 2- and 10-μM AGE-BSA and S100B treatments. These results show a concentration-dependent increase in TUNEL-positive cells in response to AGE and S100B over that with untreated, BSA, or RAGE antibody treatments alone. Apoptosis was effectively inhibited by cotreatment with RAGE antibody (RDI anti-

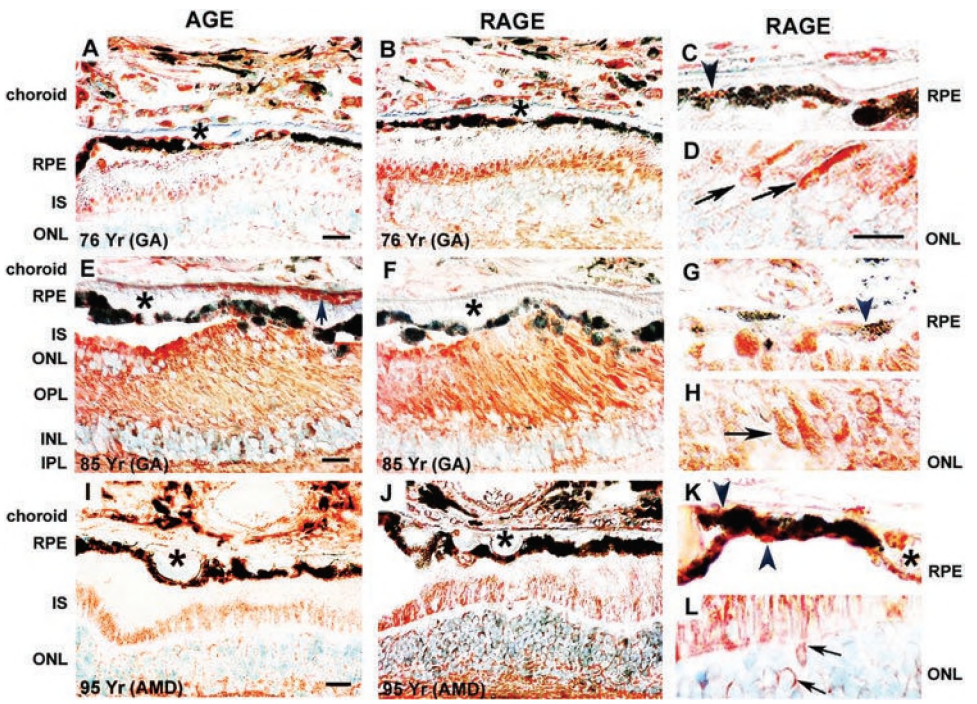

FIGURE 2. Immunolocalization of AGE and RAGE in early AMD and GA retinas. (**A**) Retinal section from a 76-year-old patient with GA and a small area of CNV (not shown in this section). AGE is labeled in RPE and photoreceptors. (✱) Drusen. (**B**) Corresponding region in an adjacent section showing RAGE labeling in RPE and IS of photoreceptors. (**C**, **D**) Higher magnification showing RAGE label in RPE (**C**, *arrowhead*) and photoreceptors (**D**, *arrows*). (**E**, **F**) Sections from an 85-year-old with GA. (✱) Drusen. (**E**) AGE labeling in basal deposits and Bruch's membrane (*arrow*). AGE labeling was also evident in RPE and remnant photoreceptors. (**F**) Corresponding section showing RAGE label in RPE and remnant photoreceptors. (**G**, **H**) Higher magnification of RAGE label in RPE (**G**, *arrowhead*) and photoreceptors (**H**, *arrow*). (**I**, **J**) Sections from a 95-year-old eye with early AMD. This region is adjacent to an area of photoreceptor cell loss. (**I**) AGE label in RPE and photoreceptors. (**J**) Corresponding region showing intense label for RAGE in RPE, IS, and ONL regions of photoreceptors. (**K**, **L**) Higher magnification of RAGE label in RPE (*arrowheads*) and photoreceptors (*arrows*). Cell nuclei were stained with methyl green. Scale bars, 25 μm.

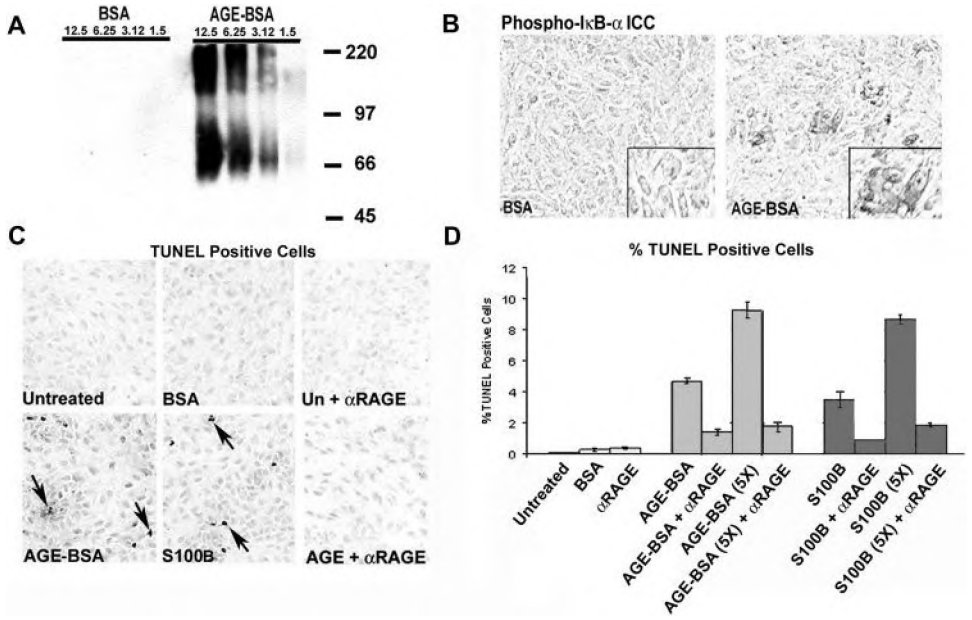

FIGURE 3. ARPE-19 cell culture experiments. RAGE-mediated effects induced by 4-day AGE-BSA and S100B treatments. (**A**) Western blot of decreasing BSA and AGE-BSA concentrations (12.5–1.5 μg) probed with the 6D12 antibody that preferentially recognizes CML-modified AGE. CML-BSA signal was found only in the AGE-BSA preparation (modified BSA monomers of approximately 66 kDa and increasing sizes, due to AGE modifications and oligomer cross-linking, were detected). (**B**) Phospho-IκB-α labeling was observed in confluent ARPE-19 cells treated with 2 μM AGE-BSA (*right*) but not with BSA (*left*). Immunopositive signal shows phosphorylated IκB-α, indicative of NFκB nuclear translocation. *Inset*: higher magnification. Nuclei were stained with methyl green. (**C**) TUNEL-positive (*arrows*) ARPE-19 cell labeling (2 μM AGE-BSA and S100B treatments of confluent cells) was prevented by anti-RAGE antibody treatments. Also note, AGE-BSA and S100B treatments induced cells to aggregate in addition to undergoing apoptosis. Nuclei were stained with methyl green. (**D**) Graph of percentage of TUNEL-positive cells after 2- and 10-μM treatments ($n = 5$, each) for BSA control and untreated wells and AGE-BSA and S100B all with or without anti-RAGE pretreatments.

hRAGE, 1:1000 dilution) in the medium. This antibody binds to the receptor extracellular ligand-binding epitope, preventing receptor interaction with AGE and S100B ligands. These results demonstrate that induction of ARPE-19 cell apoptosis by AGE and S100B ligands is mediated through RAGE.

Induction of RAGE in ARPE-19 Cells

Because AGE-BSA activated NFκB nuclear localization, treated cells were further assayed for alterations in RAGE expression by real-time RT-PCR. Primers were designed to identify only full-length RAGE mRNA and not the splice variants that do not contain all domains necessary for cell activation.[24] At 4 days of treatment with 2 μM AGE or S100B there was a sixfold increase in RAGE transcripts (Figs. 4A–C). Notably, cells treated with anti-RAGE showed inhibition of upregulation of RAGE mRNA levels. ARPE-19 cells were also assayed for AGE and S100B induction of RAGE protein (Fig. 4D). Treatments with 2 μM AGE and S100B increased RAGE protein levels, as detected by Western blot, which was not apparent with the control BSA-treated group. RAGE is differentially spliced and glycosylated among various cell types, resulting in different mobilities in SDS-PAGE and altered size detected with Western blot.[24] The RAGE antibody (C-19; Santa Cruz Biotechnology; recognizing the cytoplasmic epitope in the C terminus and specific for the full-length and shorter N-truncated isoforms) recognized a band migrating at approximately 50 kDa.[15,24] This band corresponds to the full-length RAGE protein containing the extracellular ligand binding, transmembrane, and intracellular signal transducing domains. Pretreatment with RAGE antibody (RDI) also inhibited upregulation of the full-length RAGE protein (Fig. 4D).

DISCUSSION

Although oxidative stress and RPE dysfunction are generally believed to promote disease progression in AMD, specific mechanisms governing these events are not well understood. The inherently high arterial O_2 tension environment, production of radicals in phototransduction, accumulation of photooxidative lipofuscin containing A2E in the RPE, and loss of cellular antioxidant capabilities collectively contribute to oxidative stress in the aging eye.[25–29] Correspondingly, RPE and choroidal cells alter the expression of genes for cytokines, matrix organization, cell adhesion, and apoptosis.[30–37] Chronic cellular activation perturbs normal structural and physiological integrity and may induce focal inflammatory responses at the RPE–Bruch's membrane border.[30]

The formation of AGE, such as CML and pentosidine, is accelerated in regions of oxidative stress.[6] AGE has been implicated in AMD's pathogenesis, accumulating in aging and AMD retinas at pathologic sites in Bruch's membrane and the RPE.[18–20,38] AGE formation within extracellular matrices has been proposed to contribute to diminished barrier and filtration properties in Bruch's membrane. Glycoxidation and lipoxidation reactions induced by oxidative stress have been linked to drusen formation and are proposed to contribute to the progression of AMD.[39] In this study, the additional role of AGE in RAGE-mediated cellular activation was examined as a possible contributing factor to progression.

Although a limited number of donor eyes were available for this study, AGE and RAGE immunolabeling showed a good correlation with histopathologic severity. RAGE labeling in RPE cells adjacent to drusen in otherwise normal aged maculas suggests that this may be a mechanism involved in localized damage. This is consistent with the role that RAGE-induced cellular activation plays in reinstating homeostasis—for example, in recruiting macrophages to sites of injury or inflammation. Strong immunolabeling in both RPE and photoreceptor layers in early AMD and GA maculas suggests that chronic activation through AGE/RAGE may be involved in disease progression. The appearance of AGE and RAGE in the IS region of photoreceptors was unexpected but consistently seen in various stages of disease progression associated with pathologic features and photoreceptor loss. RAGE-mediated photoreceptor responsiveness could not be directly assayed because of the lack of a suitable culture method. RPE cell cultures showed RAGE-mediated cellular activating events in response to AGE treatments,

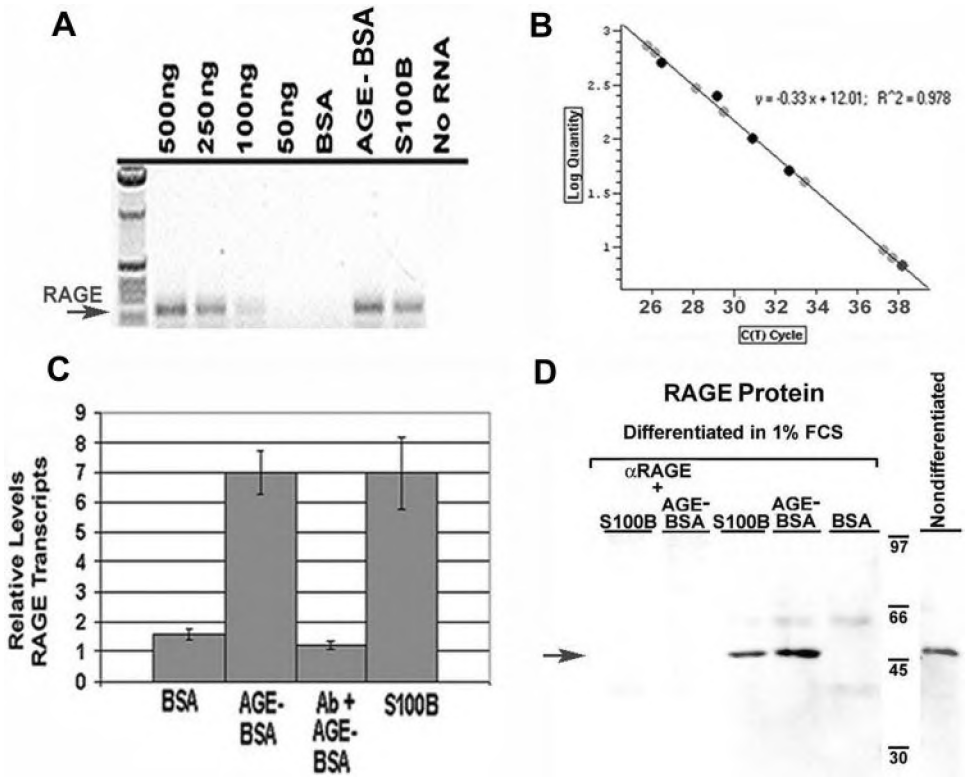

FIGURE 4. RAGE expression induced by AGE and S100B ligands in ARPE-19 cells differentiated for 2 to 3 weeks in 1% FCS. (**A**) Induction of RAGE mRNA measured by real-time RT-PCR using RAGE primers producing a 225-bp diagnostic fragment, visualized on an agarose gel (*left lane*: 1-kb ladder). The next four lanes represent amplified product using standards (500–50 ng total RNA from untreated ARPE-19 cells) after 30 cycles. The remaining lanes show amplified fragment with BSA, AGE, and S100B treatments. (**B**) Graph of fluorescence (log units) versus cycle threshold C(T) values for standards (*black symbols*) and samples (*gray symbols*). The C(T) value represents fluorescent signal above background for each reaction. The relative quantity of initial template in unknown samples can be calculated by comparison to C(T)s for standards with increasing template (example: 500 ng standard C(T) fluorescent signal is represented at 26 cycles, whereas lower template quantity 250 ng fluorescent C(T) signal occurs at approximately 29 cycles). Relationship between template quantity and number of cycles required for a significant C(T) during the exponential phase of amplification reflects an accurate calculation of template levels in a sample. (**C**) Graph of compiled data from three experiments, triplicate wells each assayed with real-time RT-PCR in triplicate reactions. (**D**) Western blot analysis showed induction of an approximate 50-kDa band with AGE and S100B treatments using the C-20 polyclonal antibody, upregulation of which are inhibited by anti-RAGE pretreatments. Note that nondifferentiated ARPE-19 cells expressed RAGE, which was downregulated in ARPE-19 cells after 2 to 3 weeks in 1% FCS (compare nondifferentiated lane with differentiated BSA-treated lane).

including NEκB nuclear localization, apoptosis, and, of importance, upregulation of the receptor. Chronic cellular activation by RAGE, altering the production of cytokines, growth factors, apoptosis mediators, and RAGE itself, is sustained by ongoing engagement of AGE ligand with the upregulated receptor.[15] Although a receptor-mediated component was not addressed, Honda et al.,[40] showed that AGE treatment of ARPE-19 cells alters a similar profile of gene expression. AGE has also been shown to induce synthesis of platelet-derived growth factor (PDGF) and VFGF in RPF cell culture.[41,42] In this context, it is important to note that RAGE has been shown to regulate growth factors and cytokines (such as TNF, VFGF, PDGF, ILs, and monocyte chemoattractant protein [MCP]-1) matrix organizing proteins (matrix metalloproteinases [MMPs]), and adhesion proteins (vascular cell [VCAM] and intercellular [ICAM] adhesion molecules) in other aging disorders.[13–17,27–29,43–46] Future studies will characterize RAGE- mediated expression of specific genes that contribute to RPE disease progression.

The 6D12 antibody has been characterized[47] and used by Hammes et al.[20] and others[18] to demonstrate AGE in diseased macular tissues. This antibody preferentially recognizes CML-AGE, an indicator of oxidative stress in tissues, and an additional irreversible glycoxidation product *N*-ε-(carboxyethyl)lysine (CEL). The heterogeneous groups of chemically modified AGE are not all equally toxic or cell activating. However, the correlative labeling of AGE and RAGE and the increased staining associated with retinal disease in this study suggest that at least some AGE in the aging retina is cell activating. The demonstrated activation of ARPE-19 cells after treatment with AGE-modified BSA further supports this conclusion. Of note, a recent study has shown that AGE-modified BSA generated and dialyzed with PBS can induce oxidative stress through trace redox active metal ions present in this biological buffer.[48] It is possible that oxidative stress in our AGE-BSA–treated ARPE-19 experiments is attributable, in part, to trace metal ions present in PBS during generation and dialysis of AGE-BSA. However, the contribution to oxidative stress in these experiments by this contaminant is thought to be minimal for the following reasons: (1) AGE-BSA was diluted in medium containing 1% ECS-containing antioxidants and metal binding molecules, (2) similar results were obtained with micromolar S100B (which was not prepared as AGE-BSA in a buffer solution), and (3) AGE-BSA effects were essentially inhibited by cotreatment with RAGE antibody. Therefore, in this study, the primary RAGE-mediated cellular-activating events were most likely induced by AGE-BSA ligands. The precise identification of the physiologically relevant AGE that induce receptor-mediated cellular activation in the aging retina awaits future studies.

Our results support a link between oxidative stress, AGE formation, and AGE induced receptor-mediated cellular activation in the aging retina. We propose that AGE induce RAGE-mediated cellular activation in RPE and photoreceptors and that this activation contributes to disease progression in AMD. Inhibitors of AGE formation and RAGE in animal models of atherosclerosis and diabetes dramatically inhibit disease progression, even in the continued presence of initiating events.[15,49] The AGE/RAGE pathway has recently become a target for intervention in these aging disorders. Targeting ligand/RAGE–mediated chronic activation, before photoreceptor and vision loss, may provide an alternative approach for intervention in the dry form of AMD.

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