Expression and Function of Receptors for Advanced Glycation End Products in Bovine Corneal Endothelial Cells

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Purpose. The corneal endothelium is a target of the aging process. This study was undertaken to reveal the relationship between corneal endothelial cell (CEC) death and the accumulation of advanced glycation end products (AGEs), by investigating the possible mechanism of accumulation of AGE in CECs and its effects on CEC death.

Methods. First, the in vivo expression of the receptor was investigated for AGE (RAGE) and galectin-3, both receptors for AGE, at both the mRNA and protein levels. Second, AGEs were added to the culture media of the cultured CECs, and the uptake of AGEs, the generation of reactive oxygen species, and the induction of apoptosis were investigated.

Results. Immunohistochemistry and RT-PCR demonstrated that both RAGE and galectin-3 were expressed in bovine CECs. After administration of AGE-modified bovine serum albumin to the culture medium, uptake of AGE was observed in the cytoplasm of the cultured bovine CECs. In addition, with increasing concentration of AGEs, the generation of reactive oxygen and the number of apoptotic cells also increased.

Conclusions. These results show that the accumulation of AGEs in CECs induced apoptosis, in part, by increasing cellular oxidative stress. The accumulation of AGEs in the CECs of elderly patients may be involved in the loss of CECs during the aging process. (*Invest Ophthalmol Vis Sci.* 2003;44:521–528) DOI:10.1167/iovs.02-0268

The corneal endothelium is a target of the aging process.[1] With increasing age, the density of the corneal endothelial cells (CECs) is known to decrease. In fact, Bourne et al.[2] estimate that CEC density decreases by 0.6% per year. Because of changes in CEC density, polymegethism and pleomorphism typically arise.[1,2] In diabetic patients, the degree of polymegethism and pleomorphism is significantly increased in CECs, even though the density of the CECs is unchanged.[3]

In addition, the normal function of CECs is altered during aging. For example, phosphatic metabolism, such as the synthesis of adenosine triphosphate (ATP)[4] and prostaglandin E_2,[5] decreases in CECs with age. Also, increased permeability of the corneal endothelium is observed with increased age; thus, the ability of CECs to function as a barrier diminishes.[1] Therefore, data associated with the aging of the corneal endothelium are accumulating; however, the mechanism or mechanisms involved are still unknown.

Advanced glycation end products (AGEs) have been proposed as common denominators for the pathogenesis of age-related diseases,[6] such as age-related macular degeneration,[7,8] atherosclerosis,[9] Alzheimer's disease,[10,11] and diabetic complications, such as diabetic retinopathy,[12–14] nephropathy,[15] and neuropathy.[16] AGEs are produced by the nonenzymatic reduction, oxidation, and condensation of reducing sugars and the ϵ-amino group or N-terminal group of proteins, collectively known as glycoxidation.[17,18] AGEs are known to modify proteins, such as bovine serum albumin (BSA) and collagen at lysine and/or arginine residues.[19] These AGE-modified proteins accumulate in tissue and cartilage with increasing age, and this accumulation is accelerated in patients with diabetes.[20] AGE modification of proteins deteriorates or eliminates their function.[21,22] In addition, AGEs are known to impair cellular function by increasing cellular oxidative stress on binding to their specific cell surface receptors, such as receptor for AGE (RAGE) and galectin-3.[23,24] The damage to proteins by modification of AGEs and the deleterious effect on cells are associated with the aging process and the pathogenesis of diabetic complications.[6]

In a previous report, we showed that formation of AGEs in the basement membrane of corneal epithelial cells plays an important role in diabetic keratopathy, a delay in the corneal epithelial wound healing process.[21] In addition, we compared accumulation of AGEs in CECs obtained from patients aged more than 50 years in whom diabetes was or was not present. However, the effect of the accumulation of AGEs and the mechanism of action on CECs remain unclear.

In the present study, we identified a possible mechanism of CEC loss, as seen in the aging process, and its association with the accumulation of AGEs. We determined the presence of AGEs in CECs and the effect they have on CECs in vitro. In addition, we investigated the expression RAGE and galectin-3 in vivo and in vitro.

Materials and Methods

Preparation of Recombinant Human Galectin-3

Recombinant human galectin-3 was prepared as described previously.[25,26] Briefly, DNA encoding for galectin-3 was inserted into the pET vector (Novagen, Madison, WI) at the *Eco*RI site, followed by

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transformation of *Escherichia coli*. Galectin-3, a 32-kDa protein, was purified from *E. coli* lysate with a galactose-conjugated Sepharose 4B column (Amersham Biosciences, Buckinghamshire, UK).[27] Partial amino acid sequencing if the protein'ss N-terminal region revealed that it is a fusion protein of 12 of the amino acids from the pET vector sequence and human galectin-3 (data not shown).

Preparation of Polyclonal Anti–galectin-3 Antibody

To raise the polyclonal antibody against human galectin-3, 1.0 mg of galectin-3 in 50% Freund's complete adjuvant was injected intradermally into a rabbit at 20 sites, followed by five booster injections with 0.5 mg of galectin-3 in 50% (vol/vol) Freund's incomplete adjuvant. Serum was collected 10 days after the final immunization, followed by purification as IgG by protein-G–conjugated affinity chromatography. The specificity of the antibody was confirmed by immunoblot assay.[26] The human galectin-3 antibody exhibited cross-reactivity with bovine galectin-3 (data not shown).

Preparation of Polyclonal Anti-RAGE

To generate an antibody specific for RAGE, a fragment of human RAGE peptide at the carboxyl terminal (E^{392}EPEAGESSTGGP^{404}) was conjugated with keyhole-limpet hemocyanin. The conjugated peptide (0.5 mg) in 50% (vol/vol) Freund's complete adjuvant was injected intradermally into 20 cutaneous sites in a rabbit, followed by three booster injections with 0.2 mg of the final immunization. Serum was collected 10 days after the final immunization, followed by purification as IgG by protein-G–conjugated affinity chromatography. The sequence of the carboxyl terminal of bovine RAGE is E^{404}EPEAAESSTGGP^{416} with more than 92% of homology to that of human RAGE. The antibody cross-reacts to bovine RAGE (data not shown).

Immunohistochemistry

Fresh bovine corneas were purchased from an abattoir. The corneas were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 15 minutes. After three washes for 10 minutes each, the corneas were embedded in optimal cutting temperature (OCT) compound (Miles Laboratories, Naperville, IL). Frozen sections, 6 μm in thickness, were cut (Kryostat 1720; Leitz, Wetzlar, Germany). After washes with PBS three times for 5 minutes each, the sections were treated with 3% hydrogen peroxide in PBS for 15 minutes to inactivate the endogenous peroxidase activity. After thorough washing, the sections were incubated with 10% normal rabbit serum for 20 minutes at room temperature to avoid nonspecific binding of the primary antibodies. The sections were then incubated overnight at 4°C with 3 μg/mL of the anti-RAGE and anti-galectin-3 polyclonal antibodies in PBS containing 1% BSA. Immunoreactivity was detected by the streptavidin-biotin-peroxidase method (Histofine SAB-PO[R] kit; Nichirei, Tokyo, Japan), according to the manufacturer's protocol. The final reaction product was visualized with 3,3′-diaminobenzidine tetrahydrochloride (DAB). Experiments were performed in triplicate.

Expression and Cloning Bovine RAGE and Galectin-3

Total RNA was isolated from fresh bovine CECs with extraction reagent (Isogen; Nippon Gene, Toyama, Japan). Reverse transcription of 10 μg total RNA was performed (Superscript II reverse transcriptase; GibcoBRL, Grand Island, NY).

A primer pair of galectin-3 was designed based on the sequence of the complete human galectin-3 cDNA in the gene bank. The sense primer was designed at the proline-glycine-alanine-tyrosine (PGAY) repeat motif, and the antisense primer at the carbohydrate recognition and binding site of galectin-3. The sense primer had a sequence of 5′-CCTATCCTGGGGCCTATCC-3′, which corresponded to nucleotides 171 to 189 of the human galectin-3 gene, and an antisense primer of 5′-GAAGCGTGGGTTAAAGTGGAAGGC-3′, which corresponded to nucleotides 519 to 542.

A primer pair for bovine RAGE was designed in accordance with a previous report. The sense primer had the sequence of 5′-CTGGATGCTAGTCCTCAGTCTG -3′, which corresponded to nucleotides 36 to 57, and the antisense primer of 5′-CCTTTGCCATCAGGAATCAGAG -3′, which corresponds to nucleotides 494 to 515.

Using the above primers, cDNA was amplified by PCR according to the after: 30 cycles of 60 seconds at 94°C, 60 seconds at 58°C, and 60 seconds at 72°C. PCR products were then subcloned into TA vectors (Invitrogen, San Diego, CA). Positive clones were confirmed by sequence analysis, with fluorescent dideoxynucleotides and an automated sequencer (model 310; Applied Biosystems, Foster City, CA).

Cell Culture

Primary cultured bovine CECs were used. Eyes from 3-year-old cattle were obtained from an abattoir, and the corneal buttons were prepared within 3 hours after enucleation. CECs with Descemet's membrane were dissected under a microscope and separated in the presence of trypsin-EDTA (GibcoBRL). Cells were cultured in 60-mm culture dishes (Falcon, Lincoln Park, NJ) in Eagle's minimum essential medium (EMEM; Gibco) with 15% fetal bovine serum (FBS) and 20 mg/L gentamicin (Gibco) in humidified atmosphere with 5% CO_2 at 37°C. Second-phase cells were used in this study.

AGE- and CML-BSA Preparation

AGE-bovine serum albumin (BSA; low-endotoxin BSA; Sigma, St. Louis, MO) was prepared by incubating 20% BSA for 30 days with 250 mM glucose-6-phosphate at 37°C in phosphate-buffered saline (PBS; pH 7.4) under sterile conditions. N^{ε}-(carboxymethyl)lysine (CML)-BSA was prepared according to a published method.[28,29] Briefly, 2 mg/mL BSA was incubated with 0.75 M glyoxylic acid (Sigma) and 0.3 M $NaCNB_3$ (Sigma) in PBS at 37°C for 24 hours. At the end of incubation, the solution was lyophilized and reconstituted with distilled water (DW). Finally, the condensed solutions were dialyzed against PBS, sterile filtered through 0.22-μm nylon filters (Pall Corp., Ann Arbor, MI).

Immunohistochemical Localization of AGE in Cultured CECs

Primary cultured bovine CECs were seeded onto sterilized, round glass coverslips of 12-mm diameter and allowed to reach 70% confluence. Then, 2 mg/mL BSA or AGE-BSA was added to the culture medium and incubated for 3 days. The localization of AGE was examined immunohistochemically.

An anti-AGE monoclonal antibody (6D12) was prepared as previously described.[30] Briefly, mice were immunized with AGE-modified albumin, and the splenic lymphocytes from the immunized mouse were fused to myeloma cells. The culture supernatant of hybrid cells was screened by its reactivity to AGEs. The selected cell lines were injected into BALB/c mice for production of ascites. The antibody was purified by protein G-Sepharose diethylaminoethyl (DEAE)-cellulose chromatography. Our recent study revealed that this antibody recognizes CML protein adducts as an epitope.

The localization of AGE in the cultured CECs was visualized using the streptavidin-biotin method. Briefly, cultured CECs on round glass coverslips were washed three times with 0.01 M PBS (pH 7.4) and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 15 minutes. After three washes in PBS for 5 minutes each, the samples were treated with 3% hydrogen peroxide in PBS for 15 minutes to inactivate the endogenous peroxidase activity. After a thorough wash, the samples were incubated with 10% normal rabbit serum for 20 minutes at room temperature to avoid nonspecific binding of the primary antibodies. The samples were incubated overnight at 4°C with 3 μg/mL of the anti-CML monoclonal antibody (6D12) in PBS containing 1% BSA. Immunoreactivity was detected by the streptavidin-biotin-peroxidase method (Histofine SAB-PO(M) kit; Nichirei), according to the manufacturer's protocol. The final reaction product was visualized

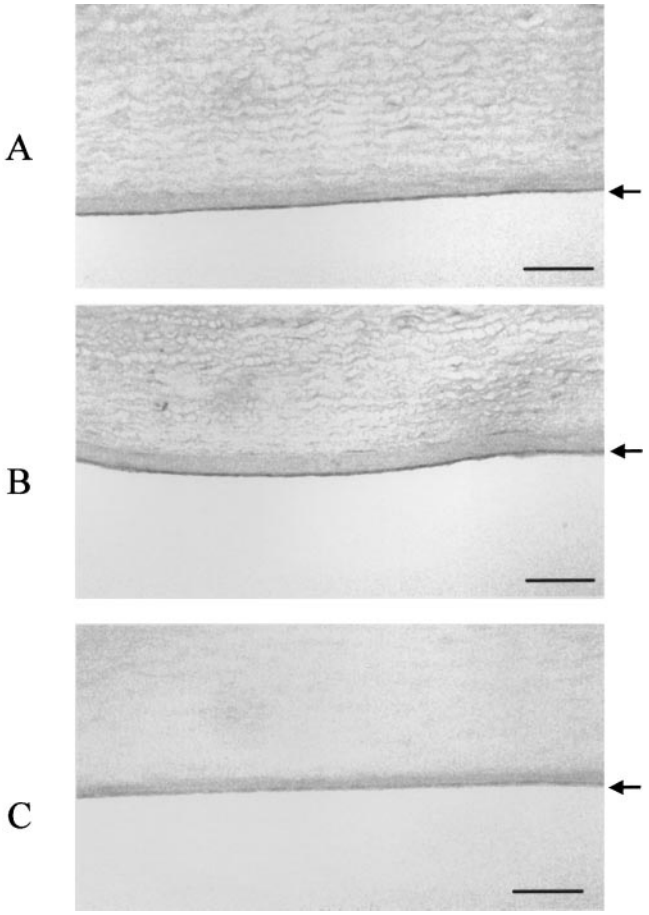

FIGURE 1. Immunohistochemical localization of galectin-3 (**A**) and RAGE (**B**) in bovine CECs. Immunoreactivity for both RAGE and galectin-3 was recognized in CECs (*arrows*) but not in corneal stroma. (**C**) No staining was recognized with control antibody (*arrow*). Bar, 50 μm.

with 3,3′-diaminobenzidine tetrahydrochloride (DAB). Experiments were performed in triplicate.

Measurement of Reactive Oxygen Species using NBT

The effect of the AGE-BSA on the formation of reactive oxygen species was analyzed using nitro blue tetrazolium (NBT) as previously described.[31] CECs (4×10^5) were cultured for 48 hours in a 12-well plate and used in the experiment. After starvation in culture medium containing 1% FBS overnight, 0 to 10,000 μg/mL of AGE-BSA or CML-BSA was added to the culture medium. After 2 days of incubation, NBT was added to the culture medium, to a final concentration of 1 mM and incubated for 60 minutes at 37°C. The concentration of reduced NBT was quantitatively measured, as previously described.[32] Briefly, the cultured cells were centrifuged at 1000*g* at 4°C for 15 minutes. After aspiration of the supernatant, 200 μL pyridine (Sigma) was added to the purple granule button and kept in boiling water for 10 minutes. The tubes were centrifuged at 500*g*, and a second extraction using 200 μL of pyridine was repeated. After centrifugation, the colored extracts were combined and read in a spectrophotometer (Spectra Max190; Molecular Devices, Sunnyvale, CA) at 515 nm. In each experiment, the content of reduced NBT in approximately 10^6 cultured bovine CECs was analyzed.

Detection of Apoptosis

To determine whether AGE induces apoptosis in cultured CECs, the number of apoptotic cells were counted after terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) staining with an in situ apoptosis detection kit (ApopTag; Intergen, Purchase, NY). The CECs were cultured on round glass coverslips and placed at the bottom of a 24-well culture dish. Cells (2×10^5) were cultured in each well for 48 hours and used for the experiment. After overnight starvation in culture medium containing 1% FBS, the nonadherent cells were washed out by gentle pipetting. Then, 0 to 10,000 μg/mL AGE-BSA or CML-BSA was added to the culture medium and maintained for 2 days. In accordance with the manufacturer's protocol for detecting apoptosis, the number of apoptotic cells was determined by light microscope. At ×100, 10 visual fields were randomly selected in each well, and the total number of cells and apoptotic cells were counted.

Statistics

The differences were calculated with a one-way fractional ANOVA test or two-way factorial ANOVA. Significance was set at $P < 0.05$.

RESULTS

Immunoreactivity for both galectin-3 and RAGE was recognized in CECs, but not in the corneal stroma (Fig. 1).

As shown in Figure 2A, PCR with cDNA from bovine CECs and primers for galectin-3 produced two fragments (330 and 240 bp) that were shorter than predicted (370 bp).

These shorter fragments were probably produced because the sense primer for galectin-3 was designed for the repeating PGAY sequence in human galectin-3. Each PCR fragment was cloned into the TA cloning vector and sequenced. Each PCR fragment showed the same 3′-end nucleotide sequence with different 5′-end sequences. The sequence of the longest PCR product corresponded to 226 to 542 nucleotides of human galectin-3. The longest PCR fragment was aligned to human galectin-3 with the highest identity of 77.0% and 76.0% at nucleotide acid and protein level, respectively (Fig. 3). In the region corresponding to the carbohydrate-recognition and

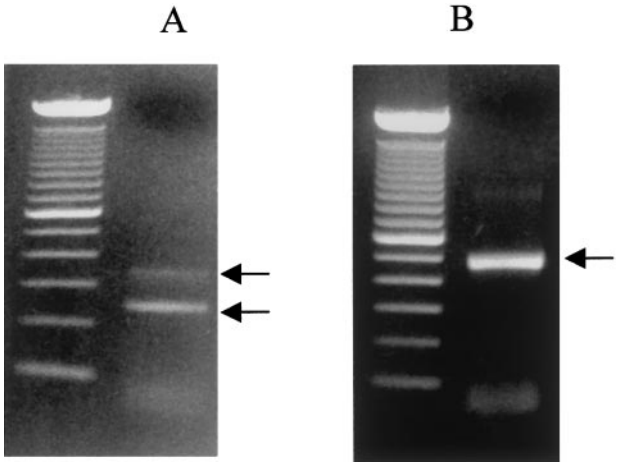

FIGURE 2. Expression of galectin-3 (**A**) and RAGE (**B**) mRNA in bovine CECs. (**A**) *Right lane*: PCR using cDNA from bovine CECs and primers for galectin-3 produced two fragments (330 and 240 bp, *arrows*) that had shorter length than predicted (370 bp). *Left lane*: molecular markers. These shorter fragments were probably produced because the sense primer for galectin-3 was for the repeating PGAY sequence in human galectin-3. Each PCR fragment was introduced into TA cloning vector and sequenced. Each PCR fragment showed the same 3′-end nucleotide sequence with different 5′-end sequences. The sequence of the longest PCR product corresponded to nucleotides 226 to 542 of human galectin-3. (**B**) *Right lane*: The mRNA expression of RAGE was detected as the band at 480 bp (*arrow*). *Left lane*: molecular markers.

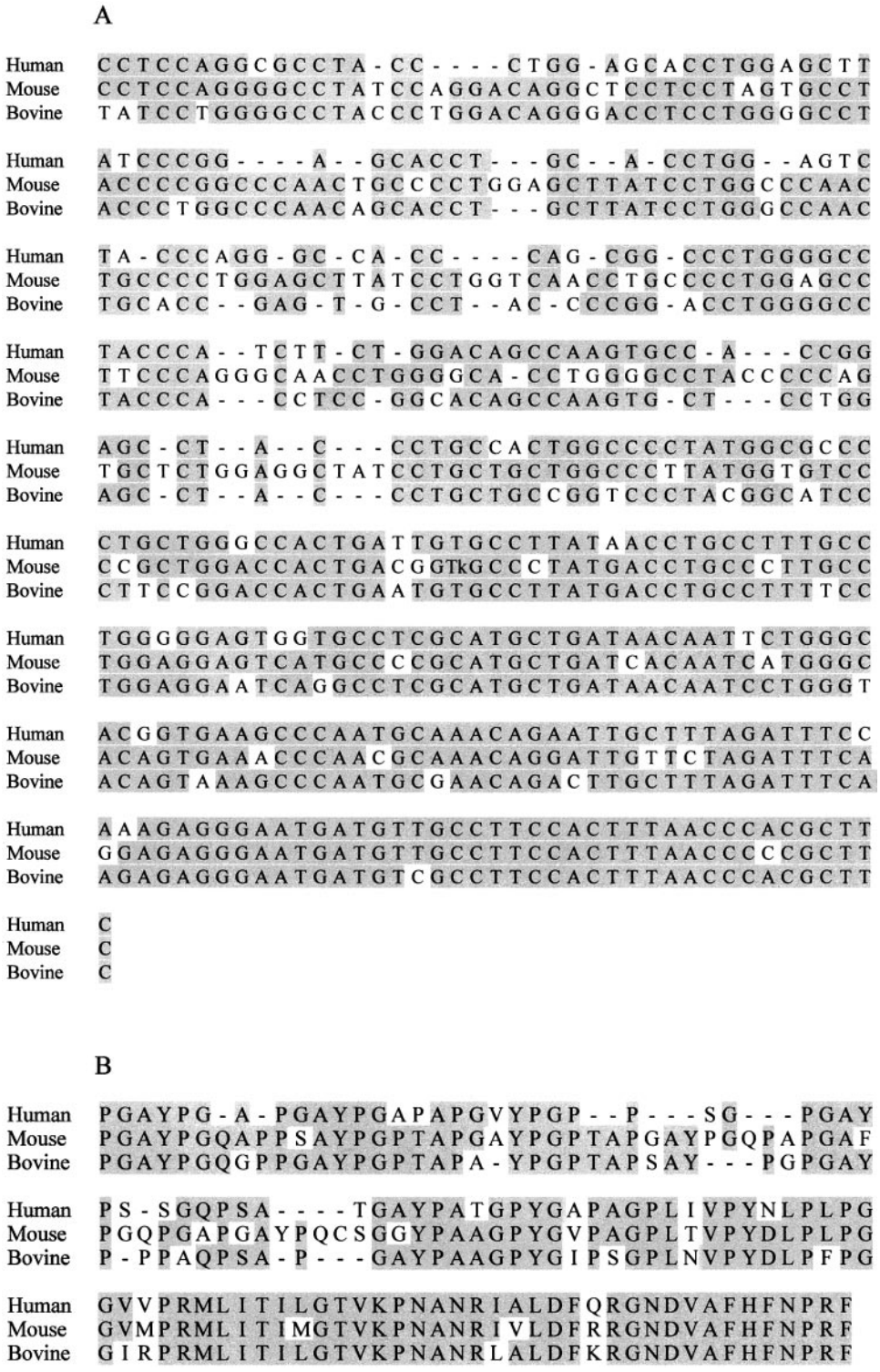

FIGURE 3. Galectin-3 gene sequence. (**A**) Alignment of human and mouse galectin-3 DNA sequences corresponding to 230 to 589 of human sequence with the bovine one, which we have disclosed on the bottom. *Shaded areas*: Identical DNA sequences; *dashes*: gaps in the sequence needed to maintain alignment. (**B**) Alignment of human and mouse galectin-3 amino acid sequences corresponding to 60 to 178 of human sequence with the bovine one in the bottom row. The bovine galectin-3 sequences were aligned to human ones with the highest identity of 77.0% and 76.0% at nucleotide acid and amino acid level, respectively.

binding sites, the identity was more than 90% at both nucleotide acid and protein level.

Expression of RAGE mRNA was noted in bovine CECs (Fig. 2B), and sequences of the PCR products corresponded to those from human RAGE (data not shown).

The localization of CML in cultured bovine CECs is shown in Figure 4. CML was not detected in CECs treated with BSA (Fig. 4A). Three days after incubation with 2 mg/mL of AGE-BSA, the immunoreactivity to CML was detected in the cytoplasm of CECs (Fig. 4B). In contrast, the immunoreactivity to CML was very weak in CECs incubated with 2 mg/mL CML-BSA (Fig. 4C).

Figure 5 shows the concentration of reactive oxygen in the cultured bovine CECs. The concentration of reduced NBT increased linearly with the addition of AGE-BSA or CML-BSA in culture medium. A significant increase in the amount of reduced NBT was detected in the concentrations of 100 to 10,000 μg/mL AGE-BSA and 250 to 10,000 μg/mL CML-BSA compared with the control (no AGE-BSA or CML-BSA). Compared with the two groups of AGE-BSA and CML-BSA, the

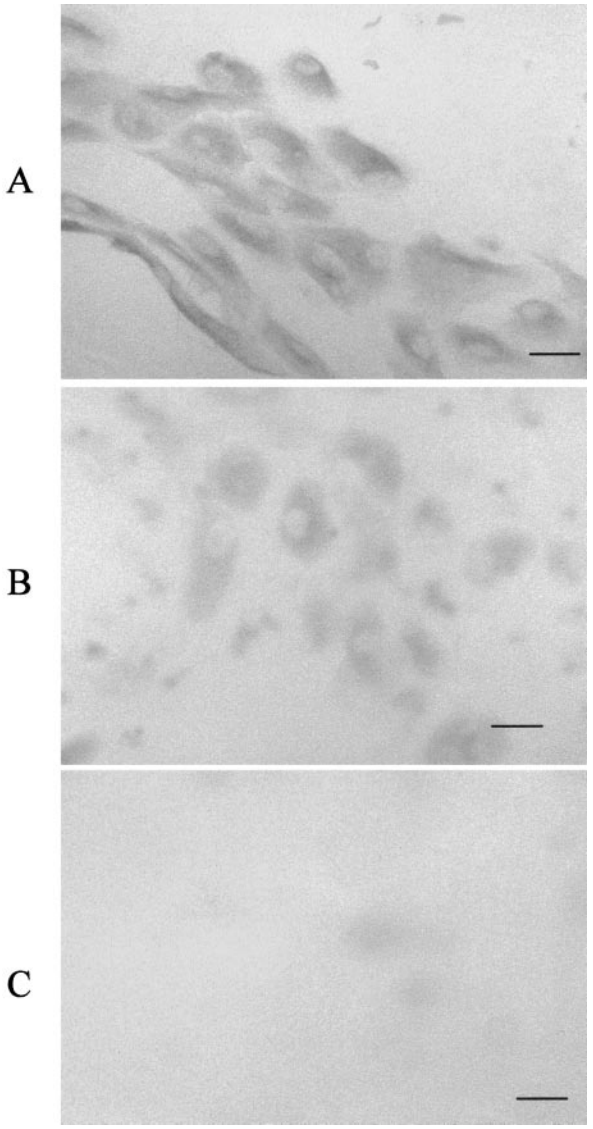

FIGURE 4. The localization of AGE in cultured bovine CECs. AGE accumulated in cytoplasm of CECs after incubation with addition of AGE-BSA to the culture medium (**A**). In the CML-BSA–treated group, the immunoreactivity to CML was very weak in the cytoplasm (**B**). No immunoreactivity was detected in the cells after addition of BSA (**C**). Bar, 20 μm.

amount of reduced NBT in AGE-BSA was significantly increased compared with that in CML-BSA (two-way factorial ANOVA, $P < 0.05$).

Figure 6A shows the rate of apoptotic CECs exposed to various concentrations of AGE-BSA or CML-BSA in the culture medium. The rate of the TUNEL-positive apoptotic cells increased with AGE-BSA and CML-BSA at concentrations of 250 to 10,000 μg/mL in the culture medium, compared with the CECs treated with BSA (Fig. 6A). AGE-BSA induced significantly more apoptosis than CML-BSA (two-way factorial ANOVA, $P < 0.05$). Figure 6B shows an example of TUNEL-positive apoptotic cells exposed to 1000 μg/mL AGE-BSA in the culture medium for 2 days. The apoptotic cells had condensed nuclei, fragmentation of chromatin, and decreased cell volume.

DISCUSSION

In the current study, the AGE receptors, RAGE and galectin-3, were expressed in the corneal endothelium in vivo. Those receptors served to incorporate AGEs into the cytoplasm, which led to the formation of reactive oxygen species and apoptosis. The concentration at which AGEs induces formation of reactive oxygen species and apoptosis varies, depending on the condition of the experiment, but included a range of 14.5 to 2000 μg/mL.[33,34] In our experiment, a significant increase in the formation of reactive oxygen species and apoptosis was observed at 100 and 250 μg/mL of AGE-BSA or higher. Those AGE concentrations are much higher than that present in the aqueous humor.[35] However, the concentration of AGEs present in the CECs in vivo may be much higher than that present in the aqueous humor, because AGEs gradually accumulate in CECs,[21] which do not turn over during a person's life. One of the purposes of the present study is to reveal the mechanism of the accumulation of AGE in CECs. The results of the study suggest that AGEs present in CECs may originate, at least in part, from AGE receptor uptake of AGEs present in the aqueous humor. However, we cannot deny the possibility that part of the AGE accumulation in corneal endothelium is from modification of intracellular proteins.

Several AGE receptors have been reported to date, such as galectin-3,[36] RAGE,[37,38] 80K-H,[39] OST-48,[39] CD36,[40] and macrophage scavenger receptors.[41] The function of the AGE receptors is not fully understood. One proposed role of AGE

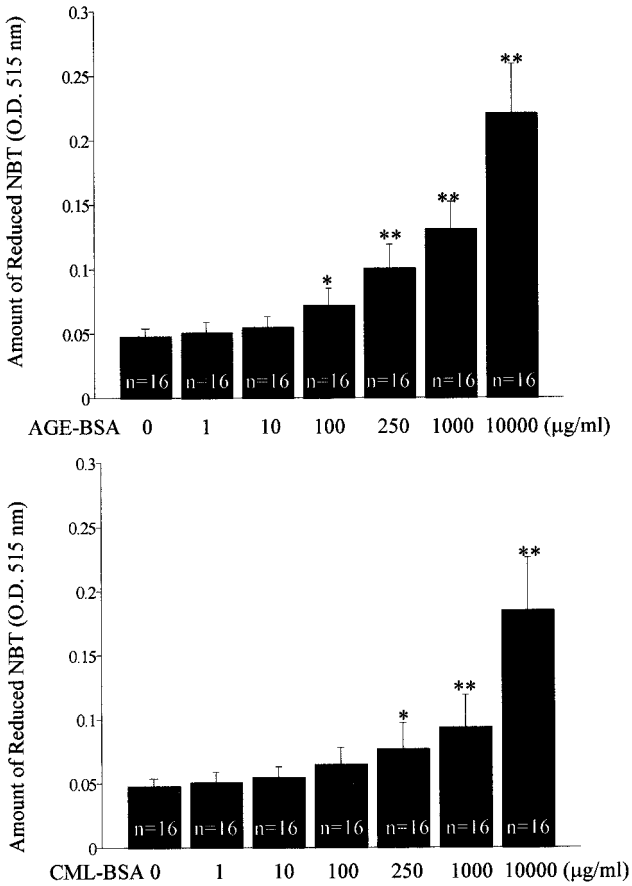

FIGURE 5. The concentration of reactive oxygen in the cultured bovine CECs in the culture medium containing various concentration of AGE-BSA (*top*) or CML-BSA (*bottom*). The concentration of reduced NBT indicates that of reactive oxygen in the cultured endothelial cells. The concentration of reduced NBT increased with that of AGE-BSA or CML-BSA. AGE-BSA and CML-BSA induced reactive oxygen formation in a dose-dependent manner. In addition, AGE-BSA induced more reactive oxygen formation than CML-BSA (two-way factorial ANOVA: $^{*}P < 0.05$, $^{**}P < 0.01$).

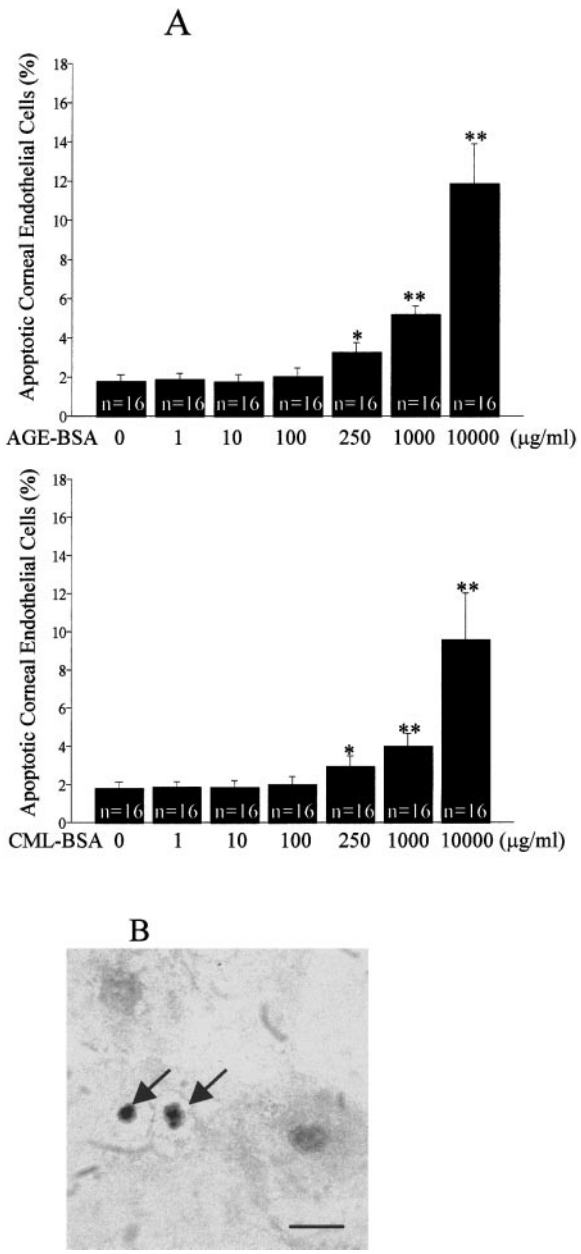

FIGURE 6. AGEs and CML induced apoptosis in cultured bovine CECs. (**A**) The number of apoptotic cells increased with the concentration of AGE-BSA or CML-BSA in the culture medium. The induction of apoptosis was quantitated by TUNEL staining. AGE-BSA and CML-BSA induced apoptosis in the cultured CECs in a dose-dependent manner. In addition, AGE-BSA induced apoptosis more than CML-BSA (two-way factorial ANOVA: $^{*}P < 0.05$, $^{**}P < 0.01$). (**B**) The apoptotic cells had condensed nuclei, fragmentation of chromatin, and decreased cell volume. Shown is an example of bovine CECs cultured in medium containing 1000 μg/mL AGE-BSA for 2 days. Bar, 20 μm.

receptors is the endocytosis of AGE-modified proteins. On endocytosis, the AGE-proteins in serum are partially digested into AGE peptides, which is easily excreted in the urine.[42] This means that AGE receptors serve as scavengers to inhibit the accumulation of AGEs in the body. Another possible role of AGE receptors is the activation of intracellular signaling pathways.[43] The interaction of AGEs and AGE receptors induces oxidative stress,[24] a reduction in the antioxidant defense mechanism,[44] activation of NF-κB,[45] the increased expression of stress cytokines,[45,46] and the induction of apoptosis.[33,47] These changes are thought to be involved in the pathogenesis of diabetic complications and the aging process.

AGEs induce oxidative stress and vice versa. In other words, an increase in oxidative stress leads to the accumulation of AGEs. Increased oxidative stress accelerates the intracellular oxidation of sugars and proteins,[17,48] which help to form AGEs and AGE-modified proteins. In addition, the presence of AGEs stimulates the expression of AGE receptors, including RAGE.[44] Taken together, these findings show that accumulation of AGEs in CECs may accelerate the intracellular generation of AGEs and the uptake of AGEs from the aqueous humor due to increased oxidative stress.

In the present study, we compared the biological effect of AGE-BSA and CML-BSA on cultured bovine CECs. AGE is a generic term used to classify various adducts of proteins and reducing sugars. In the preparation method of AGE-BSA used in the present study, 40 of 59 lysine residues were modified, of which only 9 were CML adducts.[28] Thus, AGE-BSA contains CML and non-CML adducts in the same preparation of BSA. The CML content of CML-BSA used in the present study was 9.25 mol/mol of BSA, indicating that the amounts of CML adducts of AGE-BSA and CML-BSA were similar.[29] The binding affinity of AGE-BSA and CML-BSA differs between RAGE and galectin-3.[36,43] RAGE has a high affinity to both AGE-BSA and CML-BSA, whereas galectin-3 has a high affinity to some of the non-CML adducts of AGE-BSA.[36,43] Therefore, the biological effect of CML-BSA is mediated by RAGE.

When bovine CECs were cultured in medium containing AGE-BSA for 3 days, CML was detected in the cytoplasm of these cells. In contrast, the immunoreactivity to CML was very weak when the CECs were cultured in medium containing CML-BSA. These results suggest that the interaction of galectin-3 and non-CML components of AGE-BSA is necessary for endocytosis. However, it does not mean that galectin-3 is the only receptor involved in the process of AGE endocytosis. Yamamoto et al.[49] have reported that RAGE is involved in the endocytosis of AGEs.[49] In addition, other receptors for AGEs (80K-H,[39] OST-48,[39] CD36,[40] and macrophage scavenger receptor[41]) detected, although weakly, in CECs treated with CML-BSA indicates that there are mechanisms of AGE endocytosis that are unrelated to galectin-3.

The biological effect of AGE- and CML-BSA was slightly different in NBT and TUNEL assays. Both AGE- and CML-BSA induced apoptosis and the formation of reactive oxygen in cultured CECs dose dependently. However, AGE-BSA was more effective than CML-BSA at inducing the formation of reactive oxygen and apoptosis at concentrations of 250 to 10,000 μg/mL and 1000 to 10,000 μg/mL, respectively. The difference in the biological effect between AGE- and CML-BSA seems unrelated to the number of CML adducts in the BSA molecule, because the number of CML adducts in AGE-BSA was not less than that of CML-BSA.[28,29] These results indicate that not only CML adducts but also non-CML adducts of AGE-BSA were important in inducing reactive oxygen formation and apoptosis. In addition, both galectin-3 and RAGE were involved in the biological effect of AGE-BSA.

If the accumulation of AGEs in CECs involves apoptosis, it opens the possibility that the density of CECs would be less than that in nondiabetic patients. However, the density of CECs does not differ significantly between diabetic and nondiabetic patients, although there is an increase in the degree of pleomorphism in CECs of diabetic patients.[50] These phenomena may be explained by considering the concentration of AGEs in the aqueous humor. Endo et al.[35] have reported that the concentration of AGEs in the aqueous humor is slightly higher in diabetic patients than in normal control subjects; however, Hashimoto et al.[51] have reported that there is no significant difference in the concentration of pentosidine, one

of the AGEs. This means that concentration of AGEs in CECs may depend on the age and not so much on the state of diabetes. For this reason, the decrease in corneal endothelial density is dependent on age and not on the diabetic state.

The purpose of the present study was to reveal the mechanism of CEC death during aging. Our previous study demonstrated that cultured human CECs become senescent more quickly in vitro when the cells are obtained from older donors.[52] This result indicates that CECs become senescent in vivo as the person gets older. In general, cellular senescence has been attributed to both the shortening of telomeres and/or cellular damage. However, human CECs maintain their telomere length because they do no proliferate in vivo.[53] Thus, senescence of human CECs in vivo is thought to be due to cellular damage. The current results suggest that accumulation of AGEs may be one cause of cellular damage of human CECs in vivo. Therefore, our results indicate that accumulation of AGEs plays an important role in the aging of CECs.

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References

1. Mishima S. Clinical investigations on the corneal endothelium-XXXVIII Edward Jackson Memorial Lecture. *Am J Ophthalmol.* 1982;93:1–29.
2. Bourne WM, Nelson LR, Hodge DO. Central CEC changes over a ten-year period. *Invest Ophthalmol Vis Sci.* 1997;38:779–782.
3. Larsson LI, Bourne WM, Pach JM, Brubaker RF. Structure and function of the corneal endothelium in diabetes mellitus type I and type II. *Arch Ophthalmol.* 1996;114:9–14.
4. Lass JH, Greiner JV, Merchant TE, Glonek T. The effects of age on phosphatic metabolites of the human cornea. *Cornea.* 1995;14:89–94.
5. Jumblatt MM. PGE2 synthesis and response pathways in cultured CECs: the effects of in vitro aging. *Curr Eye Res.* 1997;16:428–435.
6. Singh R, Barden A, Mori T, Beilin L. Advanced glycation end-products: a review. *Diabetologia.* 2001;44:129–146.
7. Ishibashi T, Murata T, Hangai M, et al. Advanced glycation end products in age-related macular degeneration. *Arch Ophthalmol.* 1998;116:1629–1632.
8. Hammes HP, Hoerauf H, Alt A, et al. N(epsilon)(carboxymethyl)lysin and the AGE receptor RAGE colocalize in age-related macular degeneration. *Invest Ophthalmol Vis Sci.* 1999;40:1855–1859.
9. Stitt AW, Bucala R, Vlassara H. Atherogenesis and advanced glycation: promotion, progression, and prevention. *Ann NY Acad Sci.* 1997;811:115–127; discussion 127–119.
10. Vitek MP, Bhattacharya K, Glendening JM, et al. Advanced glycation end products contribute to amyloidosis in Alzheimer disease. *Proc Natl Acad Sci USA.* 1994;91:4766–4770.
11. Yan SD, Chen X, Schmidt AM, et al. Glycated tau protein in Alzheimer disease: a mechanism for induction of oxidant stress. *Proc Natl Acad Sci USA.* 1994;91:7787–7791.
12. Beisswenger PJ, Makita Z, Curphey TJ, et al. Formation of immunochemical advanced glycosylation end products precedes and correlates with early manifestations of renal and retinal disease in diabetes. *Diabetes.* 1995;44:824–829.
13. Stitt AW, Li YM, Gardiner TA, Bucala R, Archer DB, Vlassara H. Advanced glycation end products (AGEs) co-localize with AGE receptors in the retinal vasculature of diabetic and of AGE-infused rats. *Am J Pathol.* 1997;150:523–531.
14. Hammes HP, Wellensiek B, Kloting I, Sickel E, Bretzel RG, Brownlee M. The relationship of glycaemic level to advanced glycation end-product (AGE) accumulation and retinal pathology in the spontaneous diabetic hamster. *Diabetologia.* 1998;41:165–170.
15. Raj DS, Choudhury D, Welbourne TC, Levi M. Advanced glycation end products: a nephrologist's perspective. *Am J Kidney Dis.* 2000;35:365–380.
16. Sugimoto K, Nishizawa Y, Horiuchi S, Yagihashi S. Localization in human diabetic peripheral nerve of N(epsilon)-carboxymethyllysine-protein adducts, an advanced glycation endproduct. *Diabetologia.* 1997;40:1380–1387.
17. Baynes JW. Role of oxidative stress in development of complications in diabetes. *Diabetes.* 1991;40:405–412.
18. Lyons TJ. Glycation, oxidation, and glycoxidation reactions in the development of diabetic complications. *Contrib Nephrol.* 1995;112:1–10.
19. Baynes JW, Thorpe SR. Role of oxidative stress in diabetic complications: a new perspective on an old paradigm. *Diabetes.* 1999;48:1–9.
20. Dyer DG, Dunn JA, Thorpe SR, et al. Accumulation of Maillard reaction products in skin collagen in diabetes and aging. *J Clin Invest.* 1993;91:2463–2469.
21. Kaji Y, Usui T, Oshika T, et al. Advanced glycation end products in diabetic corneas. *Invest Ophthalmol Vis Sci.* 2000;41:362–368.
22. Kaji Y, Amano S, Usui T, et al. Advanced glycation end products in Descemet's membrane and their effect on CEC. *Curr Eye Res.* 2001;23:469–477.
23. Thornalley PJ. Cell activation by glycated proteins: AGE receptors, receptor recognition factors and functional classification of AGEs. *Cell Mol Biol (Noisy-le-grand).* 1998;44:1013–1023.
24. Wautier MP, Chappey O, Corda S, Stern DM, Schmidt AM, Wautier JL. Activation of NADPH oxidase by AGE links oxidant stress to altered gene expression via RAGE. *Am J Physiol Endocrinol Metab.* 2001;280:E685–E694.
25. Hsu DK, Zuberi RI, Liu FT. Biochemical and biophysical characterization of human recombinant IgE-binding protein, an S-type animal lectin. *J Biol Chem.* 1992;267:14167–14174.
26. Zhu W, Sano H, Nagai R, Fukuhara K, Miyazaki A, Horiuchi S. The role of galectin-3 in endocytosis of advanced glycation end products and modified low density lipoproteins. *Biochem Biophys Res Commun.* 2001;280:1183–1188.
27. Levi G, Teichberg VI. Isolation and physicochemical characterization of electrolectin, a beta-D-galactoside binding lectin from the electric organ of Electrophorus electricus. *J Biol Chem.* 1981;256:5735–5740.
28. Urata Y, Yamaguchi M, Higashiyama Y, et al. Reactive oxygen species accelerate production of vascular endothelial growth factor by advanced glycation end products in RAW264.7 mouse macrophages. *Free Radic Biol Med.* 2002;32:688–701.
29. Ikeda K, Higashi T, Sano H, et al. N (epsilon)-(carboxymethyl)-lysine protein adduct is a major immunological epitope in proteins modified with advanced glycation end products of the Maillard reaction. *Biochemistry.* 1996;35:8075–8083.
30. Horiuchi S, Araki N, Morino Y. Immunochemical approach to characterize advanced glycation end products of the Maillard reaction: evidence for the presence of a common structure. *J Biol Chem.* 1991;266:7329–7332.
31. Zhang H, Agardh CD, Agardh E. Retinal nitro blue tetrazolium staining and catalase activity in rat models of diabetes. *Graefes Arch Clin Exp Ophthalmol.* 1996;234:324–330.
32. Baehner RL, Nathan DG. Quantitative nitroblue tetrazolium test in chronic granulomatous disease. *N Engl J Med.* 1968;278:971–976.
33. Min C, Kang E, Yu SH, Shinn SH, Kim YS. Advanced glycation end products induce apoptosis and procoagulant activity in cultured human umbilical vein endothelial cells. *Diabetes Res Clin Pract.* 1999;46:197–202.
34. Takeuchi M, Bucala R, Suzuki T, et al. Neurotoxicity of advanced glycation end-products for cultured cortical neurons. *J Neuropathol Exp Neurol.* 2000;59:1094–1105.
35. Endo M, Yanagisawa K, Tsuchida K, et al. Increased levels of vascular endothelial growth factor and advanced glycation end products in aqueous humor of patients with diabetic retinopathy. *Horm Metab Res.* 2001;33:317–322.
36. Vlassara H, Li YM, Imani F, et al. Identification of galectin-3 as a high-affinity binding protein for advanced glycation end products (AGE): a new member of the AGE-receptor complex. *Mol Med.* 1995;1:634–646.
37. Neeper M, Schmidt AM, Brett J, et al. Cloning and expression of a cell surface receptor for advanced glycosylation end products of proteins. *J Biol Chem.* 1992;267:14998–15004.

38. Schmidt AM, Yan SD, Brett J, Mora R, Nowygrod R, Stern D. Regulation of human mononuclear phagocyte migration by cell surface-binding proteins for advanced glycation end products. *J Clin Invest.* 1993;91:2155–2168.
39. Li YM, Mitsuhashi T, Wojciechowicz D, et al. Molecular identity and cellular distribution of advanced glycation endproduct receptors: relationship of p60 to OST-48 and p90 to 80K-H membrane proteins. *Proc Natl Acad Sci USA.* 1996;93:11047–11052.
40. Ohgami N, Nagai R, Ikemoto M, et al. CD36, a member of class B scavenger receptor family, is a receptor for advanced glycation end products. *Ann NY Acad Sci.* 2001;947:350–355.
41. Araki N, Higashi T, Mori T, et al. Macrophage scavenger receptor mediates the endocytic uptake and degradation of advanced glycation end products of the Maillard reaction. *Eur J Biochem.* 1995;230:408–415.
42. Vlassara H, Brownlee M, Cerami A. High-affinity-receptor-mediated uptake and degradation of glucose-modified proteins: a potential mechanism for the removal of senescent macromolecules. *Proc Natl Acad Sci USA.* 1985;82:5588–5592.
43. Kislinger T, Fu C, Huber B, et al. N(epsilon)-(carboxymethyl)lysine adducts of proteins are ligands for receptor for advanced glycation end products that activate cell signaling pathways and modulate gene expression. *J Biol Chem.* 1999;274:31740–31749.
44. Bierhaus A, Illmer T, Kasper M, et al. Advanced glycation end product (AGE)-mediated induction of tissue factor in cultured endothelial cells is dependent on RAGE. *Circulation.* 1997;96:2262–2271.
45. Yan SD, Schmidt AM, Anderson GM, et al. Enhanced cellular oxidant stress by the interaction of advanced glycation end products with their receptors/binding proteins. *J Biol Chem.* 1994;269:9889–9897.
46. Vlassara H, Brownlee M, Manogue KR, Dinarello CA, Pasagian A. Cachectin/TNF and IL-1 induced by glucose-modified proteins: role in normal tissue remodeling. *Science.* 1988;240:1546–1548.
47. Kasper M, Roehlecke C, Witt M, et al. Induction of apoptosis by glyoxal in human embryonic lung epithelial cell line L132. *Am J Respir Cell Mol Biol.* 2000;23:485–491.
48. Hamada Y, Araki N, Koh N, Nakamura J, Horiuchi S, Hotta N. Rapid formation of advanced glycation end products by intermediate metabolites of glycolytic pathway and polyol pathway. *Biochem Biophys Res Commun.* 1996;228:539–543.
49. Yamamoto Y, Yamagishi S, Yonekura H, et al. Roles of the AGE-RAGE system in vascular injury in diabetes. *Ann NY Acad Sci.* 2000;902:163–170; discussion 170–162.
50. Matsuda M, Ohguro N, Ishimoto I, Fukuda M. Relationship of corneal endothelial morphology to diabetic retinopathy, duration of diabetes and glycemic control. *Jpn J Ophthalmol.* 1990;34:53–56.
51. Hashimoto H, Arai K, Yoshida S, Chikuda M, Obara Y. Pentosidine and autofluorescence in lenses of diabetic patients. *Jpn J Ophthalmol.* 1997;41:274–277.
52. Miyata K, Drake J, Osakabe Y, et al. Effect of donor age on morphologic variation of cultured human CECs. *Cornea.* 2001;20:59–63.
53. Egan CA, Savre-Train I, Shay JW, Wilson SE, Bourne WM. Analysis of telomere lengths in human CECs from donors of different ages. *Invest Ophthalmol Vis Sci.* 1998;39:648–653.