Biochemical and Biophysical Research Communications **275,** 53–59 (2000)
doi:10.1006/bbrc.2000.3245, available online at http://www.idealibrary.com on IDEAL®

Effect of Advanced Glycation End Products on Lens Epithelial Cells *in Vitro*

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Received July 3, 2000

The extended exposure of proteins to reducing sugars leads to nonenzymatic glycation with the accumulation of advanced glycation end products (AGEs). Long-lived proteins, such as collagen and crystallins, are subjected to this modification, and are implicated as causal factors in several diseases including diabetic complications, cataracts, and arteriosclerosis. One means through which AGEs modulate cellular interactions is via binding to specific receptors. In the current study, the existence of AGEs in human anterior polar lens capsules of cataracts was confirmed using a combination of dot-immunoblot and fluorescent detection. Human lens epithelial cells (LECs) attached to anterior lens capsules expressed mRNA for the receptor for AGEs (RAGE). The interaction of LECs with AGEs using bovine lens epithelial explants demonstrated that AGEs induced mRNAs and proteins of fibronectin, collagen type I, aberrant extracellular matrix proteins, and α-SMA, a specific marker for myofibroblastic cells. These findings suggest that AGEs may alter cellular functions which induce mRNAs and proteins associated with fibrosis in LECs.

Key Words: **glycation; AGEs; cataracts; extracellular matrix; transdifferentiation; RAGE; bovine lens explants; fibrosis.**

With aldoses, proteins become nonenzymatically glycated through the formation of Schiff base and Amadori rearrangement products, and further undergo a complex series of reactions, such as rearrangement, cyclization, fragmentation, dehydration and oxidative modification, leading to a class of permanently modified proteins known as advanced glycation end products (AGEs). AGEs have unique properties such as fluorescence, browning, and protein cross-links [1–3]. Nonenzymatic glycation is a major modification in long-lived proteins, such as collagen [4] and crystallins [5], and one of several mechanisms that may be involved in the generation of diabetic complications [6], cataracts [7], arteriosclerosis [8], and Alzheimer's disease [9]. *In vitro* glycation of bovine lens capsules, which consists of more than 90% collagen type IV [10] markedly inhibits the basement membrane's susceptibility to matrix metalloproteinases due to the alteration of the structural and functional integrity of the extracellular matrix [11].

AGEs have been shown to be taken up through AGE specific receptors [12–14]. Among these receptors, a receptor for AGEs (RAGE), a 35 kDa protein of the immunoglobulin superfamily of cell surface molecules, has been extensively investigated. RAGE is localized in many cells and tissues where it modulates AGE effects such as activation of $p21^{ras}$, MAP kinases, NF-κB and cdc42/rac [15–17]. The interaction of RAGE with distinct ligand molecules is implicated in the inflammatory response [18], vascular perturbation [19], Alzheimer's disease [20] and tumor invasion [21].

Interactions of glomerular epithelial cells with glycated collagen type IV show reduced cell spreading, phosphorylation of focal adhesion kinase and mitogen-activated protein kinase activity, all of which suggest that the altered cell-matrix interactions induce intracellular signaling that leads to alterations in diabetic nephrology [22]. In glomerular mesangial cells, glycated BSA stimulates mRNA and/or protein levels of TGF-β1 and extracellular matrix (ECM) components [23]. The induction of TGF-β by AGEs via a receptor-mediated mechanism has been proposed in increased ECM deposition and altered cellular growth. In *in vitro* lens epithelial explants, TGF-β induces distinct anterior opacities accompanied by the formation of spindle-shaped cells that contain α-smooth muscle actin (SMA), capsule wrinkling, and deposition of abnormal extracellular matrix components, such as collagen type I (Col I) [24] which appear in human anterior subcap-

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0006-291X/00 $35.00

sular cataracts and in lens capsules which develop fibrotic changes after cataract extraction [25–27].

These findings led us to hypothesize that AGE formation in human anterior lens capsules via interaction with RAGE induces a transdifferentiation of lens epithelial cells. In the present study, we confirmed the existence of AGEs on human anterior capsules, found for the first time, induction of RAGE mRNA on anterior LECs from clinically distinct types of cataract, and showed AGE-mediated induction of mRNAs and proteins associated with transdifferentiation and ECM formation by LECs in bovine lens epithelial explants.

MATERIALS AND METHODS

Preparation of human anterior lens capsules. Lens capsules and the associated LECs were obtained from patients who underwent cataract extraction. After injecting viscoelastic material into the anterior chamber, continuous curvilinear capsulorrhexis was performed, and the anterior capsule was carefully removed with forceps. The lens capsules were immediately frozen in liquid nitrogen, and stored at −70°C for RNA preparation and protein extraction. These clinical anterior capsule samples prepared from the above extraction were used for dot blot assay, in which the sample sizes of anterior polar cataracts (AP), cortical cataracts (C) and posterior poplar cataracts (PP) were 10 each. For fluorescence detection, separate samples each from AP ($n = 3$) and C ($n = 3$) patients were used. The extents of fluorescence (mean ± SD) are representative of five independent experiments.

Solubilization of lens capsules. For a dot immunoblot assay, the human lens capsules were solubilized as described previously [28] with a slight modification. The anterior capsules were added to a solution containing proteolytic enzyme inhibitors: 1 mM phenylmethylsulfonyl fluoride, 4 μg/ml of aprotinin, 4 μg/ml of leupeptin, 4 μg/ml of pepstatin, and 2 mM EDTA. Epithelial cell debris adhering to the anterior lens capsule was separated by stirring batches of 10 capsules in 10 ml of sodium deoxycholate (20 g/liter) for 2 h at 4°C, followed by additional stirring in 10 ml of sodium deoxycholate (5 g/liter) for 2 h at 4°C. Then, the lens capsules were partially blotted dry, and suspended in 1 ml of solubilization buffer which contained 27 mM Tris, 13.4 mM H_2SO_4, 20 g/liter SDS, pH 6.1, and all of the proteolytic enzyme inhibitors. To facilitate solubilization, the capsules were sonicated at 4°C for 5 min twice in a sonicator (Branson Sonifier 250, Danbury, CT). The samples were flushed with nitrogen, sealed, and shaken at 70°C overnight. Undissolved capsules were pelleted by centrifugation (8700*g*) for 10 min at 4°C. The supernatant was collected, and the pellets were dissolved by boiling in solubilization buffer for 2 h followed by centrifugation (8700*g*) for 10 min at 4°C. The second supernatant was combined with the first one to evaluate for AGE formation in human anterior lens capsules. Protein determinations were performed using the BCA method according to the manufacturer's recommendations (Pierce, Rockford, IL).

Preparation of AGE-BSA. AGE-bovine serum albumin (BSA) was prepared by adding 5 mg of BSA (essentially fatty acid free; Sigma, St. Louis), 25 mM glycolaldehyde and 1 mM diethylenetriaminepentaacetic acid in 1.0 ml of 0.1 M phosphate buffer (pH 7.0), sterile-filtering and incubating at 37°C for up to 5 days. Unincorporated sugar was removed by dialysis against a 5 mM phosphate buffer (pH 7.0). Control BSA was exposed to 37°C for the same time interval and in the same buffer without glycolaldehyde. The concentration of AGE-BSA and BSA was determined with the BCA method.

Measurement of human anterior lens capsular AGE proteins. Equal amounts (10 μg) of solubilized proteins from the anterior lens capsules were dot blotted onto a nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, NH). AGEs were detected by using the polyclonal anti-AGE antibody, which was synthesized by injecting glycolaldehyde-incubated keyhole limpet hemocyanine into rabbits, and recognizes carboxymethyllysine and pentosidine, two well-characterized AGEs, as well as several other AGE epitopes [29]. The immnunocomplexes were visualized using an enhanced chemiluminescence (ECL) kit (Amersham International, Amersham, UK), and quantified by scanning densitometry using an ImageMaster VDS (Pharmacia Biotech Inc., Uppsala, Sweden). The assay was standardized by blotting with different amounts of AGE-BSA.

For fluorescence detection of general AGE formation, the above three LEC-free capsules which had had the epithelial cell debris detached, were digested extensively by sequential treatment with collagenase and pepsin according to the method described [30] with modifications. Anterior lens capsules from each cataract sample were re-suspended with bacterial collagenase in 50 mM Tris, 0.2 M NaCl, 2 mM $CaCl_2$, pH 7.4, at 1:100 enzyme to substrate ratio, and incubated for 18 h at 37°C. At the end of incubation, the samples were centrifuged at 70,000 rpm for 6 h. The pellets were re-suspended with pepsin in 0.1 M acetic acid and 0.02% sodium azide at a 1:150 enzyme-to-substrate ratio, and incubated for 36 h at 37°C with intermittent sonication. At the end of the incubation period, nonsolubilized material was pelleted by centrifugation (2000 rpm for 2 min), and fluorescence of the supernatant was determined to estimate AGE formation using excitation/emission wavelengths of 370/440 nm and 335/385 nm. These wavelengths detect general AGE formation [31] and pentosidine-related fluorescence [32], respectively. Readings were obtained with a spectrofluorometer (Kontron Instrument Model SFM 25, Jürich, Switzerland).

Treatment of bovine lens epithelial explants. Young bovine eyes were obtained from a local abattoir, and transported to the laboratory in a 4°C cold chamber. After the cornea, iris, and ciliary body were removed, the lens capsule was removed from the remainder of the lens with a forceps and fine scissors. The separated layer containing LECs was transferred to a 6-well plate coated with collagen type IV in Dulbecco's Minimum Essential Medium (DMEM; GibcoBRL, Grand Island, NY) containing 20% (wt/vol) fetal bovine serum (FBS; GibcoBRL). After incubation for 20 h, the fresh explants in the absence of FBS were incubated with the cell layer downward on cell culture dishes in alternatively the absence or presence of 50, 100, and 200 μg/ml of AGE-BSA as well as 10 ng/ml of TGF-β1 as a positive control. After the indicated time points, total cellular RNA and cell lysates were prepared for RT-PCR and Western blot analysis, respectively.

RNA isolation and RT-PCR. Total cellular RNA was isolated from LECs attached to anterior capsules of human lenses from AP, C, PP, and nuclear (N) cataracts, or bovine lenses using TRIZOL reagent (GibcoBRL) according to the manufacturer's instruction. One μg of RNA was reverse transcribed in a 20-μl reaction mixture by using a kit (1st strand cDNA synthesis kit; Boehringer-Mannheim Corp., Indianapolis, IN). The cDNA (0.2 to 1 μl) was amplified in a 20-μl reaction mixture. Conditions for PCR were as follows: 0.4 μM each primer, 0.2 mM deoxynucleoside triphosphate mixture (Perkin–Elmer Corp., Foster City, CA), 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, and 1.0 U of *Taq* DNA polymerase (Perkin–Elmer Gaithersburg, MD). Reaction mixtures were incubated in a thermal controller (Model PTC-100; MJ Research, Inc., Watertown, MA) for 30 to 40 cycles (denaturation at 94°C for 45 s, annealing at 60°C for 30 s, extension at 72°C for 45 s). The amounts of amplified products were analyzed using an image documentation system (ImageMaster VDS, Pharmacia Biotech Inc., Uppsala, Sweden). DNA size markers were run in parallel to validate the predicted sizes of the amplified bands (D-15 DNA marker; NOVEX, San Diego, CA). The primer sequences specific for the genes examined and the predicted product sizes are shown in Table 1.

TABLE 1

Primer Pairs and Predicted Lengths of PCR Products with Each Pair

Gene	Size (bp)	Upstream primer	Downstream primer
Bovine			
Fibronectin	735	ggtaacgaaggctccactgc	accagattcctcttatcaactg
Collagen I	387	gaaaggagagagcggcaac	tcaataccagggagacccac
β-Actin	350	aggccaaccgcgagaagatgacc	aggccaaccgcgagaagatgacc
Human			
RAGE	480	caccttctcctgtagcttca	tgccacaagatgaccccaat[34]

Western blot analysis. Bovine LECs cells were lysed on ice in lysis buffer (20 mM Tris–HCl [pH 7.5], 120 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 2 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 4 μg/ml of leupeptin, 4 μg/ml of pepstatin and 4 μg/ml of aprotinin; Sigma). After centrifugation for 10 min at 12,000*g*, the supernatant was stored at −70°C. The lysates containing 20 μg of protein were boiled for 5 min in SDS sample buffer, fractionated by SDS–polyacrylamide (8 or 10%) gel electrophoresis, transferred to a nitrocellulose membrane (Hybond; Amersham Life Science, Inc., Cleveland, OH), and incubated with polyclonal anti-bovine fibronectin (Sigma) and anti- bovine α-smooth muscle actin antibodies (Sigma) as described above.

RESULTS

AGEs are detected in human anterior lens capsule from cataract patients. The glycation of proteins that have very slow turnover such as collagen and crystallin has been extensively studied [5, 21]. Since the lens capsule is mainly composed of collagen IV [10], and the aqueous humor of cataract patients contains high levels of H_2O_2 [33], we hypothesized that AGEs form in lens capsules. Human anterior lens capsules from cataract samples were assessed for AGE formation by dot blotting equal amounts (10 μg) of solubilized anterior lens capsule from cataracts onto nitrocellulose membranes after standardizing the assay with different amounts of AGE-BSA. AP samples (16.3 AGE units/μg) contained higher amounts of AGEs than C samples (13.9 AGE units/μg) and PP samples (8.1 AGE units/μg) (Fig. 1A). Fluorescence readings of digested capsular proteins from AP samples showed 20 and 55% ($P < 0.01$) increase in fluorescence at 370/440 nm (general AGE formation) [31] and 335/385 nm (pentosidine-related fluorescence) [32], respectively, when compared to anterior capsules from C samples (Figs. 1B and 1C).

Human lens epithelial cells express RAGE mRNA. The observation of AGEs in anterior lens capsules from cataract samples prompted us to examine LECs from cataract samples for RAGE, the best characterized receptor of AGEs. Human anterior subcapsular LECs were freshly isolated from patients having clinically different types of cataracts such as AP and C, and were analyzed by RT-PCR using specific primers for human RAGE [34]. Figure 2 shows clearly that the expression of RAGE mRNA was prominent in LECs of AP compared with other subtypes of cataracts. In all cases, β-actin expression remained uniform between epithelial cell samples from different cataracts.

Induction by AGEs of fibronectin, collagen type I and α-smooth muscle actin mRNAs and proteins in LECs. In AP, LECs transdifferentiate into mesenchyme-like cells, and abnormal extracellular matrix including fibronectin (FN), collagen type I (Col I), and α-smooth muscle actin (SMA) accumulate around the fibroblast-like cells [35, 36]. The presence of AGEs in anterior lens capsules and the abundance of RAGE in LECs of AP suggested that the binding of AGEs to RAGE on LECs may influence cellular functions. We examined the effects of AGE-BSA on the transdifferentiation of LECs using the above myofibroblast markers in bovine lens epithelial explants. Figure 3A shows that AGEs as well as TGF-β1 mediated a marked increase of mRNA for FN and Col I in bovine explants within 24 h. Immunoblot analysis confirmed that AGE treatment led to an increased accumulation of FN and SMA in bovine LECs (Figs. 3B and 3C). Also significant expression of SMA was observed by TGF-β1 treatment (Fig. 3C). Because a primer sequence for bovine SMA was not available through a Web site (the Whitehead Institute for Biomedical Research, Cambridge, MA, www.genome.wi.mit.edu), the RNA level was not determined.

DISCUSSION

The results presented in this study demonstrate for the first time, to the best of our knowledge, that AGEs accumulate in anterior lens capsules of human cataracts, that RAGE mRNA is expressed by LECs from cataract specimens, and that AGEs induce the expression of myofibroblastic markers of epithelial transdifferentiation during AP cataract development. These findings implicate AGEs in the development of anterior subcapsular cataracts.

AGEs can form from compounds other than sugars, and are accelerated by oxidative stress through a process known as glycoxidation. One example is the oxidation products of ascorbate, which is present in the

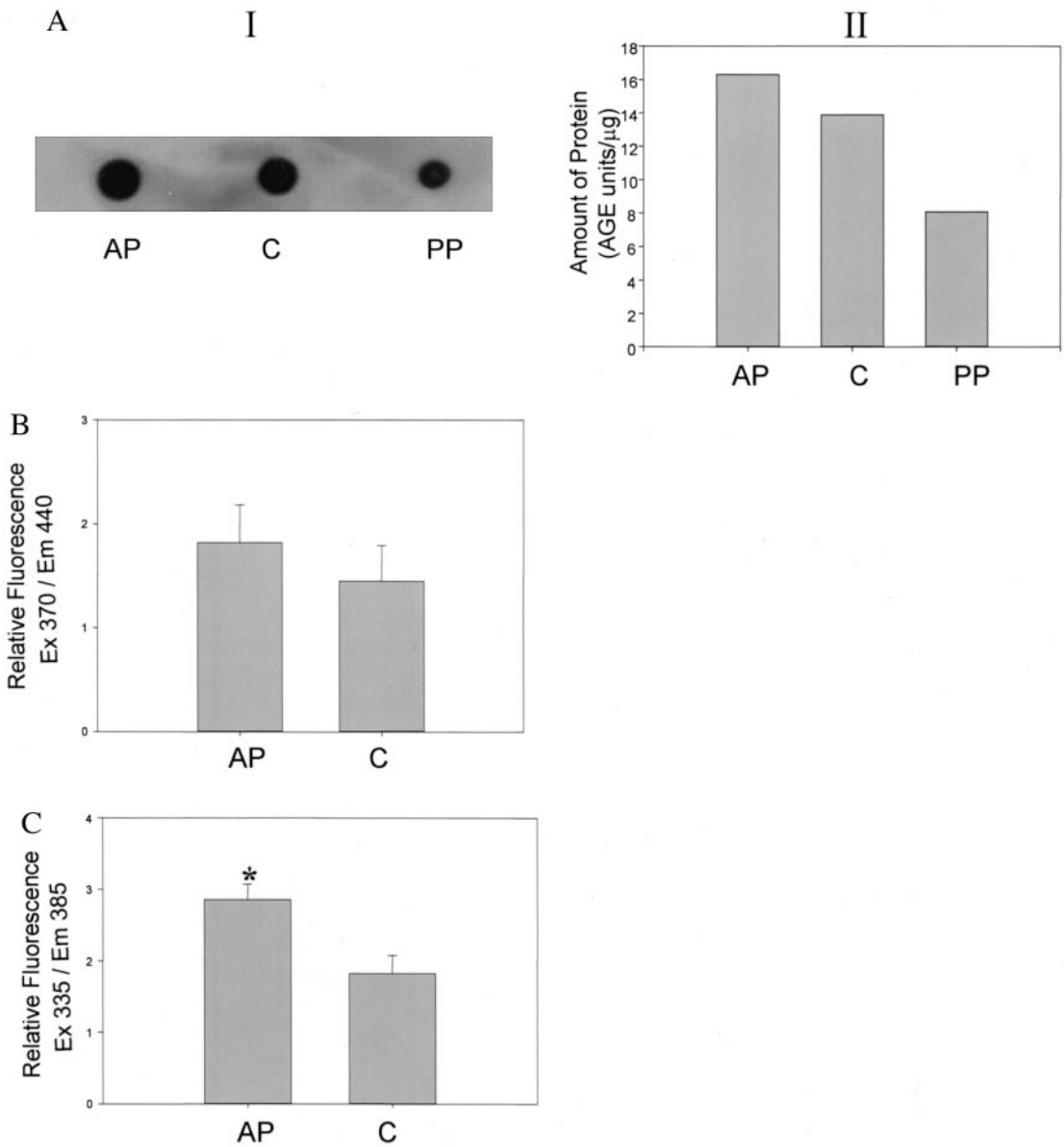

FIG. 1. Determination of AGEs in anterior polar capsules from anterior polar cataracts (AP) and cortical cataracts (C)/posterior polar cataracts (PP). (A) Capsular AGEs were determined by scanning densitometry of immunoblots; (I) Equal amounts (10 μg) of solubilized proteins from the anterior lens capsules were dot blotted onto a nitrocellulose membrane, the membrane was incubated with AGE-specific polyclonal anti-AGE antibody [29]. (II) Dot immunoblot assay was carried as described under Materials and Methods. (B) For fluorometric analysis, the three capsules were solubilized using sequential digestion with collagenase and pepsin. Fluorescence was measured at excitation/emission wavelengths of 370/440 and 335/385 nm, which detect general AGE formation and petosidine-related fluorescence, respectively. Values were corrected for the protein concentration of each sample. The extents of fluorescence (mean $\pm$ SD) are representative of five independent experiments. The *asterisk* denotes a *P* value of <0.01.

highest concentration compared to other sugars in the lens, and are highly reactive nonenzymatic glycation agents *in vitro* [37]. The concentration of ascorbate in human aqueous humor is 1.4 to 1.8 mM which is approximately 20 times higher than in plasma [38]. The oxidation of ascorbate produces several degradation products such as glycolaldehyde, glyceraldehyde and threose [39, 40]. In this study we prepared AGEs *in vitro* by incubating the reaction mixture containing BSA and glycoaldehyde, which is known to produce both carboxymethyllysine [41] and pentosidine [32], two well characterized *in vivo* glycoxidative agents. If RAGE on LECs recognizes AGEs, where do they come from? Lens capsules comprise more than 90% collagen type IV and laminin, which have a very slow turnover [42]. The level of H_2O_2 prevalent in the aqueous humor of patients with cataracts is relatively high (189 $\pm$ 88 μM) [33] which would promote oxidation of ascorbate.

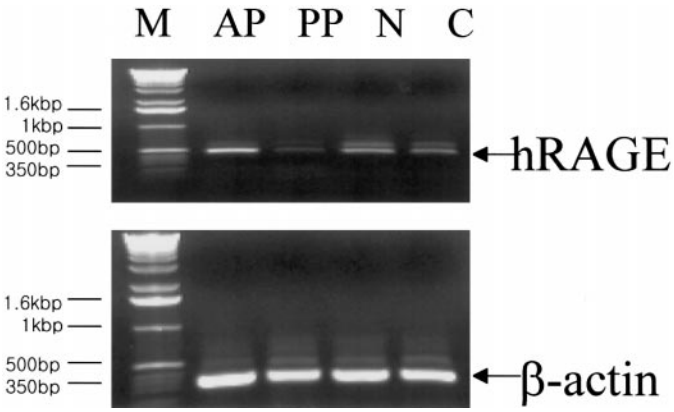

FIG. 2. Identification of human receptor for advanced glycation end products (RAGE) mRNA level in human LECs from AP, C, PP, and nuclear (N) cataracts using RT-PCR. Total cellular RNA was isolated from LECs attached to four combined anterior capsules of human lenses for each clinical types of cataract. M, molecular size standards (base pairs).

Thus, the environment for nonenzymatic glycation of these long-lived basement membrane components appears to be optimal during anterior subcapsular cataract development. In fact, our study supports this hypothesis since, AGEs in anterior lens capsules of cataracts were detected by using an anti-AGE antibody, which is capable of detecting a variety of AGE epitopes including carboxymethyllysine and pentosidine [29], and also by fluorescent analysis (Fig. 1).

Our studies detected mRNA for RAGE in LECs of cataract samples. Interestingly, LECs of AP samples showed a marked induction of mRNA RAGE compared to other types of cataract. A key signaling pathway through which AGEs induce their cellular effects requires interaction with cellular receptors, of which the most highly investigated receptor is RAGE [15, 16]. In our preliminary data, a dose-dependent activation of NF-κB by AGEs was observed in lens epithelial cell cultures suggesting that AGE-RAGE interaction may occur (data not shown). However, the possibility can not be excluded that RAGE on LECs might have other functions like a receptor for certain growth factors, because RAGE has been reported to interact with several other ligands such as amphoterin [21]. Further studies are necessary to determine the specific pathway by which AGEs alter the phenotype of LECs.

Human LECs have remarkably different morphological and immunohistochemical characteristics depending on the type of human cataract. Degenerative changes such as shrunken and vacuolated cellular matrix, and condensed nuclear chromatin are observed in LECs beneath the anterior capsule of N, while transdifferentiated changes such as spindle-shaped fibroblast-like cells embedded within a fibrillar meshwork mass are found in AP [43]. The transdifferentiation of epithelium to mesenchyme (EMT) or vice versa occurs during embryonic development [44]. During the formation of AP, and posterior capsule opacification

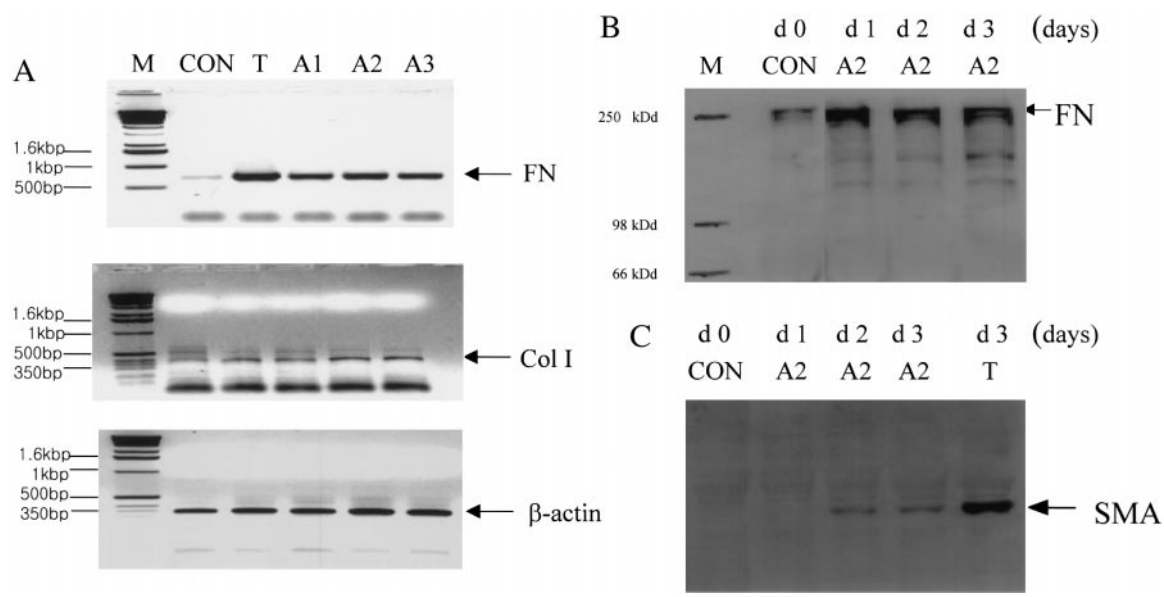

FIG. 3. Induction of AGEs of fibronectin (FN), collagen type I (Col I) and α-smooth muscle actin (SMA) mRNAs and proteins in LECs. (A) Bovine lens epithelial explants were freshly prepared and cultured with or without AGEs (A1, 50 μg/ml; A2, 100 μg/ml; A3, 200 μg/ml) or with TGF-β1 (10 ng/ml). After 20 h, total RNA was isolated, and subjected to RT-PCR analysis to determine mRNA expression of extracellular matrix proteins. At the indicated time points, cell lysates were prepared from cultured bovine lens epithelial explants and assessed for FN accumulation (B) and SMA expression (C). Data shown are from one of two independent experiments that produced similar results. M, molecular size standards (base pairs, A; kilodaltons, B and C); CON, control cells; A, cells cultured with AGEs (A1, 50 μg/ml; A2, 100 μg/ml; A3, 200 μg/ml); T, cell cultured with TGF-β1 (10 ng/ml).

that develops after cataract surgery, LECs transdifferentiate into fibroblast-like cells via the EMT process [27]. Abnormal extracellular matrix components, such as FN and Col I deposit around the transdifferentiated cells [24], and a cytoskeletal component SMA is localized in spindle-shaped cells in AP. Our experiments show that AGEs induce FN mRNA and protein, Col I mRNA, and SMA protein, which are established markers of EMT. Thus, our results suggest that AGEs mediate at least some of this phenotypic change to myofibroblasts during AP.

One pathway by which AGEs induce myoblastic transdifferention is via a mechanism involving Transforming growth factor-β (TGF-β). A multifunctional cytokine, TGF-β promotes EMT in many cell types including mammary epithelial cells [45] resulting in collagen and FN mRNA accumulation and SMA protein in LECs [35]. The overproduction of TGF-β and extracellular matrix by their sustained gene expression via autocrine control of TGF-β is proposed to be an underlying mechanism of AP formation [34]. Glycated BSA increases mRNA and protein levels of TGF-β_1 and FN, an ECM component in mesangial cell cultures, and the production of TGF-β_1 by AGEs is hypothesized to promote mesangial matrix expansion in diabetic nephropathy [23, 46]. In this regard, it would be worthwhile to determine in a future study, whether the observed enhancement of mRNA and protein levels of ECM markers in bovine LEC explants is altered by the increased active TGF-β levels by AGEs.

This study confirmed the development of AGEs in lens capsules, the existence of RAGE on LECs, and the effects of AGEs on LECs *in vitro* by which relevant inductions of mRNAs and proteins associated with transdifferentiation in bovine lens epithelial explants. However, this observation needs to be repeated with whole lenses in organ cultures, and further studies are necessary to clarify the mechanism by which AGEs interact with RAGE causes the transdifferentiation of LECs. In addition the characterization of which AGE moieties are responsible for the alteration of cellular responses in LECs is required.

ACKNOWLEDGMENTS

The authors thank Dr. B. Ortwerth, University of Missouri, Department Ophthalmology, Columbia, Missouri for valuable comments and critical review of the manuscript, and H.-K. Park, S. J. Lee, and J.-H. Kim for their technical assistance. This research was funded by National Resech Laboratory grant from Ministry of Science and Technology of Korea.

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