Published in final edited form as:

Glycoconj J. 2016 August ; 33(4): 631–643. doi:10.1007/s10719-016-9686-y.

AGE-RAGE interaction in the TGF β 2-mediated epithelial to mesenchymal transition of human lens epithelial cells

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Abstract

Basement membrane (BM) proteins accumulate chemical modifications with age. One such modification is glycation, which results in the formation of advanced glycation endproducts (AGEs). In a previous study, we reported that AGEs in the human lens capsule (BM) promote the TGF β 2-mediated epithelial-to-mesenchymal transition (EMT) of lens epithelial cells, which we proposed as a mechanism for posterior capsule opacification (PCO) or secondary cataract formation. In this study, we investigated the role of a receptor for AGEs (RAGE) in the TGF β 2-mediated EMT in a human lens epithelial cell line (FHL124). RAGE was present in FHL124 cells, and its levels were unaltered in cells cultured on either native or AGE-modified BM or upon treatment with TGF β 2. RAGE overexpression significantly enhanced the TGF β 2-mediated EMT responses in cells cultured on AGE-modified BM compared with the unmodified matrix. In contrast, treatment of cells with a RAGE antibody or EN-RAGE (an endogenous ligand for RAGE) resulted in a significant reduction in the TGF β 2-mediated EMT response. This was accompanied by a reduction in TGF β 2-mediated Smad signaling and ROS generation. These results imply that the interaction of matrix AGEs with RAGE plays a role in the TGF β 2-mediated EMT of lens epithelial cells and suggest that the blockade of RAGE could be a strategy to prevent PCO and other age-associated fibrosis.

Keywords

posterior capsule opacification; basement membrane; epithelial to mesenchymal transition; AGEs; ENRAGE; RAGE

Introduction

The ocular lens is surrounded by a capsule, which is a basement membrane (BM) secreted by the lens epithelial cells. Similar to other BMs, the proteins in this tissue have a low turnover rate and thus accumulate post-synthetic modifications with age [1]. Among the prominent modifications are those derived from oxidation and glycation. Glycation is the reaction of carbohydrates containing reactive aldo and keto groups with the amino groups of

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Conflict of interest

The authors declare that they have no conflict of interest.

proteins (mostly lysine and arginine residues) to form structurally heterogeneous compounds that are collectively known as advanced glycation endproducts or AGEs [2,3]. Thus, AGE formation in proteins is directly linked to the concentration of reactive carbohydrates. Furthermore, AGE levels in proteins are inversely related to the turnover rate of proteins, and thus the BM proteins with a low turnover rate are expected to accumulate AGEs. We reported recently that AGEs accumulate in the human lens capsule with aging and at a higher rate in the capsules of cataractous lenses [4].

Cataracts are a major cause of blindness throughout the world. In 2010 nearly 94 million people were visually impaired from cataracts globally and this number is predicted to double by 2020 [5,6]. Exposure to UV light, malnutrition, diabetes and age are all risk factors for this debilitating disease. The only remedy at present is the surgical removal of cataracts and replacement with an artificial intraocular lens (IOL). Although IOL implantation restores vision to a large extent, a problem frequently encountered by this procedure is the posterior capsule opacification (PCO) or secondary cataract formation. Thirty to forty percent of patients develop this complication within 2–5 years of cataract surgery [7].

During PCO formation, the epithelial cells remaining in the anterior capsule after cataract surgery proliferate and move along the capsule behind the implanted IOL to the posterior capsule where they transdifferentiate into mesenchymal-type cells. This process is known as the epithelial to mesenchymal transition or EMT [8,9]. The transdifferentiated cells deposit extracellular proteins and synthesize α SMA, causing wrinkling and opacity of the posterior capsule. These events lead to PCO, which impedes vision. To restore vision, Nd:YAG laser treatment is performed to remove the fibrotic tissue and clear the visual axis. The Nd:YAG laser treatment has side effects, including an increase in intraocular pressure (IOP), glaucoma, IOL damage, corneal edema, uveitis, cystoid macular edema and retinal detachment, although at a very low incidence [10,11]. Thus, efforts are underway to prevent PCO formation.

TGF β 2 in the eye is elevated in the aqueous humor of eyes after cataract surgery [12,13]. Many studies have shown that TGF β 2 is a strong inducer of EMT in lens epithelial cells [14–16]. TGF β 2 binds to its receptor on the cell surface and activates the canonical Smad-mediated pathway and minor non-canonical pathways mediated by the ERK, MAPK and Notch pathways [17–20]. These signaling pathways promote the synthesis of several EMT-associated proteins, including α SMA, vimentin and fibronectin, and suppress other proteins, such as E-cadherin and zonula occludin-1 (ZO-1) [14].

Tissue AGEs have been shown to promote EMT in kidney and lung epithelial cells [21,22]. We previously showed that AGEs in the lens capsule promote the TGF β 2-mediated EMT of lens epithelial cells [4]. Because cataract surgery is mostly performed in the elderly and their capsule proteins are expected to contain relatively high levels of AGEs, we proposed that PCO formation after cataract surgery could be due, at least partially, to AGEs in the capsule, based on our findings.

Recent studies suggest that AGEs bind to a cell surface receptor known as RAGE. RAGE belongs to the immunoglobulin family of receptors. It is a pattern-recognition receptor and

contains extracellular V, C1 and C2 domains, a transmembrane domain and a cytosolic tail [23,24]. Ligands such as EN-RAGE (S100A12) and S100B bind the V and C1 domains, while S100A6 binds V and C2 and amyloid-beta binds the V domain [25–30]. AGEs bind mostly to the V domain [31,32]. The interaction of AGE-RAGE leads to the activation of NF- κ B, as well as the activation of MEK and MAP kinases [33]. These AGE-RAGE-mediated signaling pathways have been linked to micro- and macrovascular complications of diabetes, as well as to Alzheimer's disease and cancer [34–36]. In the eye, RAGE has been implicated in diabetic retinopathy and age-related macular degeneration [37,38]. The AGE-RAGE interaction has been shown to increase intracellular oxidative stress by activation NADPH-oxidase, a key mediator in superoxide radical production [39]. Recognition that the AGE-RAGE interaction could play a role in disease pathologies has led to efforts to block that interaction, which led to the identification of sRAGE, a truncated soluble form of RAGE that binds to circulating AGEs, and the development of RAGE specific antibodies [40] and inhibitors for RAGE [41]. Furthermore, EN-RAGE, also known as S100A12, has been identified as an endogenous ligand that binds to the V and C1 domains of RAGE [25,26]. Based on these observations, we hypothesized that capsule AGEs could bind to RAGE in lens epithelial cells during the TGF β 2-mediated EMT, which was tested in the present study.

Materials and Methods

Cell culture

Human lens epithelial cells (FHL124) were a kind gift from Dr. Michael Wormstone, School of Biological Sciences, University of East Anglia, UK. This cell line was originally developed by Dr. John Reddan at Oakland University, MI. These cells were authenticated by short tandem repeat (STR) profiling. Fifteen short tandem repeat (STR) loci plus the gender determining locus, Amelogenin, were amplified using the PowerPlex® 16 HS System from Promega and processed using the ABI Prism® 3100-Avant Genetic Analyzer. Data analyzed using the GeneMapper® v4.0 Software from Applied Biosystems confirmed that the cell line was of a human male origin and showed a 96% match with the reported analysis [42] (Table 1). The cells were cultured in MEM (Life Technologies, Carlsbad, CA) containing 5% FBS (Life Technologies) and gentamycin/L-glutamate (Sigma-Aldrich, St. Louis, MO) and maintained in a humidified incubator at 37 °C containing 5% CO₂. Cells at passages 14 to 20 were used for all of the experiments.

TGF β 2 treatment

Unless otherwise indicated, FHL124 cells (1×10^5) were seeded on AGE-modified or unmodified BME in 10-cm plates in 5% FBS-MEM and maintained for 2–3 days (to reach 70–75% confluence). The medium was changed to serum-free medium before treatment with 10 ng/ml TGF β 2 (Sigma-Aldrich, Cat#SRP3170) for 24 h.

Transfection of RAGE

A GFP-RAGE plasmid (a kind gift from Dr. Alan Stitt, Centre for Vision & Vascular Science, Queens University Belfast, UK) or empty vector (pcDNA 3.1) was transfected into the FHL124 cells using Lipofectamine LTX reagent (Life Technologies, Cat#15338-030) as

per the manufacturer's protocol. Briefly, 5×10^4 cells were seeded on 35-mm petri plates 24 h before transfection. The DNA-Lipofectamine LTX complex was prepared by mixing 1 ml of MEM (without serum/antibiotics) containing the GFP-RAGE plasmid (10 μ g) and 20 μ l of Plus reagent with 1 ml of MEM (without serum/antibiotics) containing 20 μ l of Lipofectamine reagent. This complex was incubated at room temperature for 5 min and added to the plates containing the cells. After 24 h of transfection, the cells were used in experiments.

AGE modification of BME

BME is a commercially available basement membrane purified from the mouse Engelbreth-Holm-Swarm tumor (Trevigen, Gaithersburg, MD). Laminin, collagen IV, entactin and heparin sulfate proteoglycan are the major components of BME. AGE modification of BME was carried out as previously described [4]. Briefly, after coating the plates with BME (50 μ g/ml), the plate was incubated at 37 °C with a glycation mixture, which consisted of 2 mM ascorbate (Sigma-Aldrich), 25 mM glucose (Sigma-Aldrich) and 250 μ M methylglyoxal (Sigma-Aldrich), for one week at pH 7.4 under aseptic conditions. We have previously reported that this glycation procedure produced AGEs in BME, such as CML, MG-H1, MODIC etc. [4].

Treatment with RAGE antibody or EN-RAGE

Cells (1×10^5) were seeded in 10-cm plates and maintained in 5% FBS-MEM until 70–75% confluent. The medium was changed to serum-free medium 24 h prior to the treatment of cells with the RAGE neutralizing antibody (10 μ g/ml, R & D Systems, Minneapolis, MN, Cat#AF1145) or EN-RAGE (10 μ g/ml, R & D Systems, Cat#1052-ER) for 4 h. After this treatment, the cells were seeded on AGE-modified or unmodified BME. To confirm the binding of EN-RAGE, treated cells were washed three times with 1X PBS and the cell lysate (prepared using RIPA buffer) was analyzed by Western blotting for EN-RAGE.

Quantitative Real-Time PCR

RNA was extracted from the cells using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA, Cat#74134). The RNA was reverse transcribed to generate cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, Cat#205311). qPCR analysis was performed for the EMT-associated genes using a CFX Connect Real-time PCR system (Bio-Rad Laboratories, Hercules, CA). The primers used are listed in Table 2.

Western blotting

Whole cell lysates were prepared using the Mammalian Protein Extraction Reagent (Thermo scientific, Rockford, IL) containing a 1:100 diluted protease and phosphatase inhibitor cocktail (Sigma-Aldrich) (for α SMA, Fibronectin, phosphorylated and total Smad2 and ERK1/2) or with RIPA buffer (Cell Signaling Technologies, Danvers, MA) containing 1 mM PMSF (Sigma-Aldrich) (for RAGE and EN-RAGE detection). Proteins (10 to 20 μ g) were separated on 12% SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were incubated at 4 °C overnight with primary antibodies against the following proteins: RAGE (1 μ g/ml, R & D Systems), EN-RAGE (1 μ g/ml, R & D Systems, Cat#AF1052),

α SMA (1:5000 dilution, Sigma-Aldrich, Cat#A5228), Fibronectin (1:200 dilution, Santa Cruz Biotechnology, Dallas, TX, Cat#sc9068), phosphorylated (phospho S245/S250/S255, 1:1000 dilution, Cell Signaling, Cat#3104) Smad2, total Smad2 (1:1000 dilution, Cell Signaling, Cat#5339), phosphorylated (phospho T202/Y204, 1:1000 dilution, Cell Signaling, Cat#4370) ERK1/2, total ERK1/2 (1:1000 dilution, Cell Signaling, Cat#9212) and β -Actin (1:1000 dilution, Cell Signaling, Cat#4970). Appropriate HRP-conjugated secondary antibodies (1:5000 dilution, Cell Signaling) were used. The protein bands were detected using the SuperSignal West Pico or Femto Kit (Pierce Chemicals, IL).

Immunocytochemistry

Immunocytochemistry was performed as previously described with slight modifications [4]. Briefly, 35 mm culture plates were coated with BME and AGE-modified as described above. FHL124 cells were seeded and cultured on AGE-modified or unmodified BME until the cells were 80% confluent. The cells were deprived of serum for 24 h before TGF β 2 treatment (10 ng/ml) or RAGE transfection. After the respective treatments, the cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature, followed by three washes with PBS. The cells were then permeabilized with ice cold 80% methanol in PBS for 15 min at -20°C . After being blocked with 5% normal goat serum in PBS, the cells were incubated overnight at 4°C with an antibody against α SMA (1:500 dilution, Sigma-Aldrich, Cat#A5228) or RAGE (5 $\mu\text{g/ml}$, R & D Systems), followed by 1 h of incubation at 37°C with an Oregon Green 488 goat anti-mouse IgG (1:250 dilution, Life Technologies, Cat#O-6380) (for α SMA) and Texas Red Donkey anti-goat IgG (1:250 dilution, Life Technologies, Cat#PA1-28662) (for RAGE). The cells were permanently mounted with DAPI/Vectashield (Vector Laboratories, Burlingame, CA) for observation of the nuclei.

ROS measurement

ROS levels in cells were measured with H₂DCFDA as per manufacturer's protocol with slight modifications. Briefly, 0.75×10^4 cells per well were seeded on 96 well tissue culture plates and allowed to attach overnight. Cells were washed once with serum free phenol red free MEM (SF/PRF media) and treated with 5 μM CM-H₂DCFDA (Life technologies, Cat#C-6827) in SF/PRF media for 45 min in dark at 37°C . Cells were again washed once with SF/PRF media and treated with TGF β 2 as before for 2 h. Fluorescence was measured at Ex490 nm/Em522 nm in a Biotek Synergy 4 Microplate reader (Biotek, Winooski, VT). The fluorescence measured in cell-free mixture of H₂DCFDA and media served as blank.

Statistics

The data are presented as the mean $\pm$ SD of the specific number of experiments indicated in the figure legends. The data were analyzed using StatView 5.0 software (SAS Institute Inc., NC). The statistical significance was evaluated by ANOVA followed by Fisher's protected least-significant difference test, and the differences were considered significant at $p < 0.05$.

Results

AGEs promote a TGF β 2-mediated EMT response in FHL124 cells

In our previous study, we showed that AGEs in BM promoted the TGF β 2 mediated EMT response in primary human lens epithelial cells [4]. In this study, we assessed if FHL124 cells responded similarly to AGEs and TGF β 2. The mRNA levels of the EMT markers were in general higher in cells grown on AGE-modified BME when compared with cells on unmodified BME (Fig. 1a). For example, upon TGF β 2 treatment, there was a 2-fold increase ($p < 0.0005$) in the mRNA levels of α SMA in cells cultured on BME. This effect was further enhanced (2.5-fold) in cells cultured on AGE-modified BME (compared to the cells cultured on unmodified BME). Similarly, the TGF β 2-downregulated miR levels were further downregulated by AGE modification of BME ($p < 0.0005$). These results suggest that FHL124 cells are similar to primary lens epithelial cells in their response to TGF β 2 and AGEs. It is interesting to note that the levels of Smad7, which is considered to be an inhibitor for Smad signaling, were higher in TGF β 2 treated cells. Western blotting analysis also showed that cells cultured on AGE-modified BME expressed significantly higher levels of α SMA ($p < 0.05$) and fibronectin ($p < 0.0005$) upon TGF β 2 treatment compared to the cells cultured unmodified BME (Fig. 1b).

RAGE in FHL124 cells

Our results showed that RAGE was present in FHL124 cells and its levels were similar in cells grown on unmodified and AGE-modified BME. Furthermore, the RAGE levels did not change upon TGF β 2 treatment on either of these substrates (Fig. 2a).

Forced expression of RAGE stimulates the TGF β 2-mediated EMT in FHL124 cells

We next evaluated if RAGE had a role in AGE-mediated enhancement in the EMT of lens epithelial cells. FHL124 cells were transfected with GFP-RAGE; the transfection was confirmed by Western blotting and immunofluorescence (Fig. 2b, 2c). The cells transfected with RAGE showed a marked increase in the TGF β 2-stimulated EMT response compared with the cells transfected with the empty vector. After TGF β 2 treatment, the mRNA levels of α SMA were 5.6-fold higher ($p < 0.0005$) in cells cultured on AGE-modified BME compared with cells cultured on unmodified BME (Fig. 3). This response was further enhanced to 7.2-fold in cells transfected with GFP-RAGE ($p < 0.0005$). Interestingly, the levels of α SMA in TGF β 2-treated and GFP-RAGE-transfected cells were similar to cells transfected with the empty vector and cultured on unmodified BME. Similar responses were also seen for the other EMT markers. Furthermore, the TGF β 2-mediated downregulated miR184 and miR204 were further downregulated ($p < 0.0005$) on AGE-modified BME; these effects were amplified in GFP-RAGE-transfected cells cultured on the same matrix. Even though TGF β 2 treatment significantly reduced the levels of TGF β R2, RAGE overexpression did not have any significant effect on it. Together, these results suggest that AGE-RAGE interaction is likely involved in the AGE-TGF β 2-stimulated EMT response of FHL124 cells.

Blockade of RAGE attenuated the TGF β 2-mediated EMT response

FHL124 cells were treated with a RAGE antibody before they were cultured on AGE-modified and unmodified BME and treated with TGF β 2. The mRNA levels of α SMA in the antibody-treated cells were 2-fold lower ($p < 0.0005$) than in the untreated cells cultured on AGE-modified BME (Fig. 4). Interestingly, treatment with the antibody did not have any significant effect on the mRNA levels of α SMA in TGF β 2-treated cells cultured on unmodified BME. A similar pattern was seen for CTGF, another TGF β 2-upregulated gene. After TGF β 2 treatment, there was an 11-fold decrease ($p < 0.0005$) in the levels of miR204 in cells compared with the untreated controls cultured on AGE-modified BME, which was partially but significantly prevented ($p < 0.0005$) by the antibody treatment. Although a trend toward prevention was seen for miR184 and TGF β R2, it was statistically insignificant. These results indicate that the blockade of RAGE with a neutralizing antibody can reverse the TGF β 2-mediated EMT response. In the above two experiments (Fig. 3 and 4), the basal levels of α SMA but not CTGF were higher in the cells cultured on AGE-modified BME when compared to the cells cultured on unmodified BME, the reasons for this are not known.

Treatment with EN-RAGE ameliorated the TGF β 2-mediated EMT in FHL124 cells

Next, we used EN-RAGE, a ligand for RAGE, to test the role of the AGE-RAGE interaction in the TGF β 2-mediated EMT. After TGF β 2 treatment, the mRNA levels of α SMA were 3-fold higher ($p < 0.0005$) in cells compared with untreated cells cultured on AGE-modified BME (Fig. 5a). However, prior treatment with EN-RAGE reduced this to a 2.2-fold change ($p < 0.0005$). A similar pattern was seen for CTGF. Moreover, treatment with EN-RAGE did not alter the mRNA levels of α SMA or CTGF in the TGF β 2-treated cells cultured on unmodified BME. Interestingly, upon EN-RAGE treatment, a decrease in the levels for the miRs and TGF β R2 was significantly reduced ($p < 0.0005$) in cells cultured on the AGE-modified matrix. Western blot analysis showed the presence of EN-RAGE in EN-RAGE treated cells, confirming the binding of EN-RAGE to cells, possibly to RAGE (Fig. 5b). Furthermore, the FHL124 cells cultured on AGE-modified BME showed very little α SMA staining, whereas cells cultured on similar conditions but treated with TGF β 2 showed significantly higher levels of α SMA. This effect was significantly reduced when these cells were pretreated with EN-RAGE (Fig. 5c). Taken together, these results show that treatment with EN-RAGE inhibits the TGF β 2-mediated EMT in lens epithelial cells.

Treatment with EN-RAGE reduced Smad signaling in FHL124 cells

The above results suggested that binding of AGEs to RAGE was likely involved in the TGF β 2-mediated EMT of FHL124 cells. Because TGF β 2 signals through Smads, we investigated the effect of EN-RAGE treatment on Smad signaling. The western blot results showed that the pSmad2 levels increased by 1.5-fold after a 2 h treatment with TGF β 2 in cells cultured on unmodified BME, but it took 24 h for a comparable increase in cells cultured on AGE-modified BME, suggesting a slower response to TGF β 2 in the latter (Fig. 6a). Remarkably, at 24 h, the cells treated with EN-RAGE prior to TGF β 2 treatment showed 1.6-fold lower pSmad2 levels ($p < 0.0005$) when compared with untreated cells on the same matrix. There was no difference in pSmad2 between EN-RAGE-treated and untreated cells

cultured on unmodified BME. We also determined the effect of EN-RAGE treatment on phosphorylation of ERK. We did not detect a significant change in the phosphorylation of ERK across the treatment groups (Fig. 6b). These results indicate that the AGE-RAGE interaction is integral to the TGF β 2-mediated EMT in FHL124 cells and the signaling occurs through the Smad dependent pathway.

Treatment with EN-RAGE reduced ROS levels in TGF β 2 treated FHL124 cells

Previous studies have shown that AGE-RAGE interaction increases cellular oxidant stress [39]. We therefore investigated the effect of EN-RAGE treatment on ROS levels in FHL124 cells. There was a 2.4-fold increase in the ROS levels after 2 h treatment with TGF β 2 in cells compared with untreated cells cultured on unmodified BME. Cells cultured on AGE-modified BME and treated with TGF β 2 showed a significant 3-fold increase in the levels of ROS compared to the untreated cells. Intriguingly, in EN-RAGE pre-treated cells cultured on AGE-modified BME TGF β 2 treatment showed only a 2.4-fold increase in the ROS levels compared to the untreated cells ($p < 0.005$) (Fig. 7). This translates to ~21% decrease in the ROS levels when compared to the EN-RAGE untreated cells (both cultured on AGE-modified BME). These results suggest that EN-RAGE treatment inhibits TGF β 2-mediated ROS production through AGE-RAGE interaction in FHL124 cells.

Discussion

The purpose of the present study was to investigate the following: 1) whether RAGE is present in human lens epithelial cells; 2) the effect of BM-AGEs and TGF β 2 on RAGE levels; 3) whether the AGE-RAGE interaction is necessary for the enhancement of the TGF β 2-mediated EMT response in lens epithelial cells. We detected RAGE in FHL124 cells; however, its levels were unaltered by TGF β 2 treatment. This was in contrast to a previous study that showed TGF β 1 upregulating RAGE in rat hepatic stellate cells [43].

Previous studies have shown that AGEs upregulate TGF β levels in cells [44–47] and in experimental animals [48]. In addition, RAGE activation by its ligand binding leads to the synthesis of TGF β 1 [44,49]. Whether AGEs upregulated TGF β 2 by these mechanisms in lens epithelial cells is not known but it is possible. If it does occur, these events could amplify the TGF β 2 effects on lens epithelial cells.

We found that the RAGE levels were unaffected by AGEs in BME, in contrast to others who have observed the upregulation of RAGE levels by AGEs [44,50]. However, most of these studies used exogenous AGE preparations dissimilar to the BME-bound AGEs in our study. Nonetheless, our study showed that the AGE-RAGE interaction plays a role in the TGF β 2-mediated EMT of lens epithelial cells. However, in the absence of a direct effect of either TGF β 2 or BM-AGEs on RAGE levels, it is safe to assume that the AGE-induced heightened effects of TGF β 2 are due to enhanced TGF β 2 signaling. In support of this notion, we found that treatment with EN-RAGE decreased TGF β 2-induced Smad2 phosphorylation, implying decreased TGF β 2 signaling. EN-RAGE is a ligand for RAGE and therefore its binding was expected to initiate signaling via RAGE. However, our results indicated that treatment with EN-RAGE blocked the AGE-enhanced TGF β 2-mediated signaling in FHL124 cells,

suggesting that EN-RAGE antagonizes the AGE-RAGE mediated enhancement in TGF β 2's effects.

The fact that TGF β 2-upregulated pSmad2 levels were reduced by EN-RAGE treatment suggests that TGF β 2-mediated signaling is influenced by the engagement of AGEs with RAGE. In support of this notion is the observation in a previous study that showed AGEs activating the TGF β signaling in rat kidney and vascular cells [51], although the mechanisms were not investigated in that study. Signaling of TGF β 2 occurs through a heterotetrameric complex of receptor I (T β RI) and II (T β RII) (which occurs after TGF β 2 binds to T β RII)[52]. T β RII is a constitutively active kinase receptor that, after TGF β binding, phosphorylates serine/threonine residues and activates the receptor kinase activity of T β RI. The activated T β RI recruits and activates receptor-regulated Smads, which is mediated by the Smad anchor for receptor activation (SARA). T β RI phosphorylates Smad2/3, which dissociate from T β RI. The pSmad2/3 complex then binds to Smad4 and translocates to the nucleus, where it promotes or suppresses protein expression during EMT through transcriptional regulators. The enhancement of the EMT response through the AGE-RAGE interaction could be due to the promotion of any of these events in TGF β 2 signaling. Further studies are needed to understand the mechanisms.

Our studies suggest that AGEs, through binding to RAGE, enhance TGF β 2 mediated EMT in lens epithelial cells, which could promote post-cataract fibrosis in lens epithelial cells. In support of such a mechanism, other studies have found a role for AGEs and RAGE in fibrosis of renal tubular epithelial and mesangial cells, hepatic stellate cells and cardiomyocytes [49,53–57]. In addition, glycated albumin has been shown to promote α SMA and fibronectin synthesis in kidney proximal tubular cells [58]. AGEs also have been shown to promote the endothelial to mesenchymal transition [59]. However, despite these studies showing a pro-fibrotic role for RAGE, there are studies that show the anti-fibrotic properties of RAGE or that a lack of RAGE is protective against bleomycin-induced lung fibrosis [60,61]. Therefore, the role of RAGE in fibrosis could be tissue-specific and/or dependent on the fibrosis causative factor(s). In addition, AGE-RAGE signaling activates NF- κ B and ROS production in cells [62], which could contribute to the TGF β 2-mediated EMT, analogous to previous observations that have shown that these factors playing a role in EMT and fibrosis [63–65]. While RAGE antagonists FPS-ZM1 and TTP488 have been used to block amyloid- β binding to RAGE in a mouse model of Alzheimer's disease [41,66], our study showed that a RAGE ligand EN-RAGE could be used to block the TGF β 2-mediated EMT in lens epithelial cells. Thus, our study opens a potentially new avenue for blocking PCO in humans.

Acknowledgments

This work was supported by the National Institutes of Health Grants EY022061, EY023286 (to RHN). We thank Drs. Rooban Nahomi, Johanna Rankenberg and Stefan Rakete for critical reading of the manuscript.

Abbreviations

AGEs advanced glycation endproducts

BM	basement membrane
BME	basement membrane extract
EMT	epithelial to mesenchymal transition
FBS	fetal bovine serum
MEM	minimum essential medium
PCO	posterior capsule opacification
RAGE	receptor for advanced glycation endproducts
ROS	reactive oxygen species
αSMA	alpha smooth muscle actin
CTGF	connective tissue growth factor
TGFβ2	transforming growth factor beta2
ZO-1	zona occludin-1

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Figure 1a

Figure 1b

Fig. 1. AGE-modification of BME promotes the TGF β 2-mediated EMT in FHL124 cells
Epithelial cells (FHL124) were cultured on AGE-modified or unmodified BME then treated with 10 ng/ml TGF β 2 for 24 h in serum-free medium. The mRNA levels of the EMT-associated proteins were quantified by qPCR. Western blot analysis was carried out for α SMA and fibronectin with whole cell lysate (after 48 h of TGF β 2 treatment-10 ng/ml) using the respective primary antibodies as mentioned in Materials and Methods. Densitometric analyses are shown in the bar graph. The bars represent the mean $\pm$ SD of three independent experiments. NS = not significant

Fig. 2. RAGE is present in FHL124 cells

Cells were cultured and treated with TGF β 2 for 48 h as in Fig. 1 and cell lysate was prepared using RIPA buffer, and a western blot confirmed the presence of RAGE in FHL124 (a). The western blot confirmed the presence of GFP-RAGE in FHL124 cells post-transfection (b). The images shown are representative of three independent experiments. RAGE was detected using a RAGE polyclonal goat IgG and a Texas Red-conjugated donkey anti-goat IgG; DAPI/ Vectashield was used for nuclear staining. Magnification 20 $\times$ /40 $\times$ (c). Scale bar = 50 μ m. The fluorescence intensity was measured using Nikon Elements AR analysis software (Nikon Instruments Inc., Melville, NY) and the intensity plot is shown (d). The contrast of all images was enhanced to the same level for better visualization of RAGE.

Fig. 3. Overexpression of RAGE in FHL124 cells enhances the TGFβ2-mediated EMT response
FHL124 cells were cultured on AGE-modified or unmodified BME and transfected with GFP-RAGE as mentioned in the Materials and Methods. Twenty-four hours after transfection, the cells were treated with 10 ng/ml TGFβ2 for 24 h in the SF medium. The mRNA levels of the EMT-associated proteins were measured by qPCR. The bars represent the mean ± SD of three independent experiments. NS = not significant

Fig. 4. The AGE-enhanced EMT response is inhibited by treatment with a RAGE neutralizing antibody

FHL124 cells were treated with a RAGE antibody (10 µg/ml) for 4 h, cultured on AGE-modified or unmodified BME and then treated with 10 ng/ml TGFβ2 for 24 h. Quantitative PCR was carried out on the EMT-associated proteins. The bars represent the mean ± SD of three independent experiments. NS = not significant

Figure 5a

Figure 5b

Figure 5c

Fig. 5. Treatment with EN-RAGE suppresses the AGE-enhanced EMT response

FHL124 cells were treated with EN-RAGE, a ligand for RAGE, at 10 $\mu\text{g}/\text{ml}$ for 4 h in SF medium. After this, the cells were cultured on AGE-modified or unmodified BME and treated with TGFβ2 (10 ng/ml) for 24 h in SF medium. qPCR was used to measure the mRNA levels of the EMT-associated proteins and miRs (a). The bars represent the mean $\pm$ SD of three independent experiments. Western blot was carried out for EN-RAGE (b) using a polyclonal antibody (details are given in Materials and Methods). Expression of αSMA was detected using a monoclonal antibody against αSMA and a goat anti-mouse IgG – Oregon Green 488 conjugate (c). Scale bar = 50 μm . The images shown are the representative from three independent experiments. NS = not significant

Figure 6a

Figure 6b

Fig. 6. Treatment with EN-RAGE reduces the TGF β 2-mediated phosphorylation of Smad2
 FHL124 cells were treated with EN-RAGE for 4 h, followed by culturing on AGE-modified and unmodified BME and then treatment with TGF β 2 (10 ng/ml) for various time intervals shown in the figure. Whole cell lysate was prepared as described in the Materials and Methods. The effect of EN-RAGE on Smad2 (a) and ERK1/2 (b) phosphorylation in cells cultured on BME and AGE-modified BME is shown. Densitometric analyses from three independent assays (mean $\pm$ SD) are shown in the bar graphs. un=untreated control. NS = not significant

Fig. 7. Treatment with EN-RAGE lowers the ROS levels in TGFβ2-treated cells
 Cells were or untreated with EN-RAGE (10 μg/ml) cultured on AGE-modified or unmodified BME, and treated with 10 ng/ml of TGFβ2 for 2 h. The ROS generated was measured using H₂DCFDA. The bars represent the mean ± SD of three independent experiments. NS = not significant.

Table 1

Short Tandem Repeat (STR) profiling report for FHL 124.

Marker	Allele 1	Allele 2
Amelogenin	X	Y
CSF1PO	10	
D13S317	11	12
D16S539	9	11
D18S51	17	18
D21S11	28	30
D3S1358	14	17
D5S818	11	
D7S820	10	12
D8S1179	15	
FGA	21	24
Penta D	8	9
Penta E	7	11
TH01	6	9.3
TPOX	8	9
vWA	16	17

Table 2

List of primers used for qPCR.

Gene	Forward Primer	Reverse Primer	Reference
hsa-miR204	5'-ACACTCCAGCTGGGTTCCCTTTGTCATCCT-3'	5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAGGCATAG-3'	[17]
hsa-miR184	5'-ACACTCCAGCTGGGTGGACGGAGAACTGAT-3'	5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGACCCTTAT-3'	[17]
α SMA	5'-TTCAATGTCCCAGCCAT GTA-3'	5'-GAAGGAATAGCCACGCTCAG-3'	[17]
GAPDH	5'-GTCAGTGGTGGACCTGA CCT-3'	5'-TGCTGTAGCCAAATTCGTTG-3'	[17]
Smad7	5'-AAAGTGTTCCCTGGTTTCTCCATCAAGGC-3'	5'-CTACCGGCTGTTGAAGATGACCTCCAGCCAGCAC-3'	[67]
CTGF	5'-AACTATGATTAGAGCCAACTGCCTG-3'	5'-TCATGCCATGTCTCCGTACATCTTC-3'	[67]
Fibronectin	5'-CTGGAACCGGGAACCGAATATA-3'	5'-TTCTTGTCCTACATTCGGCGG-3'	[68]
TGF β R2	5'-AAGATGACCGCTCTGACATCA-3'	5'-CTTATAGACCTCAGCAAAGCGAC-3'	[68]