Age-Related Changes of Cystatin C Expression and Polarized Secretion by Retinal Pigment Epithelium: Potential Age-Related Macular Degeneration Links

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Submitted: September 10, 2013
Accepted: January 11, 2014

Citation: Kay P, Yang YC, Hiscott P, Gray D, Maminishkis A, Paraoan L. Age-related changes of cystatin C expression and polarized secretion by retinal pigment epithelium: potential age-related macular degeneration links. *Invest Ophthalmol Vis Sci.* 2014;55:926–934. DOI:10.1167/iovs.13-13239

Purpose. Cystatin C, a potent cysteine proteinase inhibitor, is abundantly secreted by the RPE and may contribute to regulating protein turnover in the Bruch's membrane (BrM). A cystatin C variant associated with increased risk of developing AMD and Alzheimer's disease (AD) presents reduced secretion levels from RPE. The purpose of this study was to analyze the effects of age and the accumulation of advanced glycation end-products (AGEs) on the expression and secretion of cystatin C by the RPE.

Methods. Confluent monolayers of human fetal RPE (hfRPE) cells were cultured using an in vitro model mimicking extracellular AGE accumulation. Cystatin C expression, secretion, and its polarity were analyzed following culture on AGE-containing BrM mimics (AGEd versus non-AGEd). Monolayer barrier properties were assessed by transepithelial resistance measurements. The relative level of cystatin C protein expression in human RPE in situ was assessed immunohistochemically in relation to age.

Results. Advanced glycation end product–exposed RPE monolayers presented significantly decreased cystatin C expression and secretion. Basolateral secretion was fully established by week 8 in non-AGEd conditions. In AGEd cultures, polarity of secretion was impaired despite maintenance of physiological barrier properties of the monolayer. In the macula region of RPE/choroid segments from human eyes, the level of cystatin C protein was reduced with increasing donor age.

Conclusions. Exposure to AGEs reduces expression of cystatin C and affects its normal secretion in cultured RPE. Age-related changes of cystatin C in the RPE from the posterior pole may compromise its extracellular functions, potentially contributing to AMD pathogenesis.

Keywords: cystatin C, secretion, aging, retinal pigment epithelium

The RPE forms a highly polarized monolayer essential for controlling spatial and temporal localization of extracellular proteins on its apical (neuroretinal) and basolateral (Bruch's membrane [BrM]/choroidal) sides.[1] The majority of RPE cells maintain a nonproliferative state[2,3] and a relatively long lifespan, as does the RPE underlying support matrix, the BrM. This longevity makes them susceptible to a myriad of age-related changes that can affect essential cellular processes. Obtaining a greater understanding of the aging process as a predisposing factor has become a key area of interest in the study of AMD, a disease associated with significant changes in the secreted proteome of the RPE.[4]

Cystatin C is among the most abundantly expressed proteins of the RPE (within the top 2% of all expressed genes in both human and mouse studies).[5–8] The precursor of this cysteine proteinase inhibitor contains an N-terminal signal peptide of 26 amino acids that is essential for targeting it to the endoplasmic reticulum (ER)/Golgi classical secretory pathway.[9,10] A polymorphism in the signal sequence (Ala25Thr due to a G73A substitution) results in an alternate homologue, variant B, homozygosity for which was reported to be correlated with an increased risk of exudative AMD development.[11] Notably, the same autosomal recessive allele was extensively documented in relation to increased risk for developing Alzheimer's disease in the Caucasian population.[12,13] The single amino acid change leads to mistrafficking of the protein away from the ER to the mitochondria,[14] likely owing to reduced hydrophobicity of the signal sequence,[15] and subsequently results in significantly decreased secretion compared with the wild type.[14] The role of cystatin C in the RPE has not yet been fully characterized, yet its high expression and secretion, coupled with its specific inhibitory properties on cysteine proteinases (cathepsins B, H, L, and S)[16,17] suggest a role in matrix remodeling and turnover, processes often prone to malfunction during AMD progression.

Protein modification described by Maillard reactions is a common phenomenon during the aging process, resulting in advanced glycation end-product (AGE) formation.[18] These

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adducts are the result of reactions between ε-amino groups and glucose, α-oxaloaldehydes, and lipid peroxidation products.[18] Advanced glycation end products accumulate on long-lived structural proteins such as collagen,[19] where, by altering their structure and function, they can contribute to the development of age-related disease.[20] Advanced glycation end-product accumulation occurs in the BrM in the eyes of aging patients[21–23] and is associated with AMD development.[24–27] Exposure to AGEs has been shown to alter the gene expression profile of cultured RPE cells, subsequently leading to altered functional capacity.[23,28,29]

In the present study, we analyzed the effects of the accumulation of AGEs and of increasing age on the expression and polarized secretion of cystatin C. We showed that following exposure of RPE cells to AGEs, both expression and secretion of cystatin C were reduced, while the polarity of its secretion was impaired. The results were corroborated by the reduction of the relative level of cystatin C protein expression with increasing age in the macula region in situ. We propose that the age-related decline of extracellular cystatin C plays a role in the imbalance of proteolytic activities on the basal side of RPE, which may contribute to the mechanisms underlying AMD pathogenesis.

Methods

Cell Culture

Human fetal RPE (hfRPE) cells were extracted and cultured as previously described.[30] Human fetal RPE cells obtained from two separate donors (donors 1 and 2) were used in the majority of experiments. In some cases, cells obtained from a third donor (donor 3) were required. Culture media for hfRPE cells (MEM, α modification) and media supplements were obtained from Sigma-Aldrich (Poole, UK), with N2 supplement (Life Technologies, Paisley, UK) replacing N1 supplement. Cells were maintained in 25-cm^2 flasks at 37°C in 5% CO_2 from time of receipt (passage 0) to no more than passage 2. Fresh media were added to flasks three times per week, and monolayers were analyzed on a weekly basis to ensure cells retained their "cobblestone" morphology and pigmentation.

AGE Modification of Extracellular Matrix (ECM) and Seeding of hfRPE Cells

All hfRPE experiments were carried out in either standard 24-well cell culture plates, or in 12-well Transwell plates (12-mm diameter polyester inserts, 0.4-μm pores; Sigma-Aldrich). Wells were coated overnight with human placental ECM (BD Biosciences, Oxford, UK) to attain a final concentration of 10 μg/cm^2. To induce an accumulation of AGEs within the ECM (from here on referred to as AGEd ECM), wells were then incubated with 100-mM glycolaldehyde (Sigma-Aldrich) at 37°C for 4 hours, followed by thorough washing with PBS. Neutralization of remaining aldehyde groups was achieved by incubating ECM with 50 mM sodium borohydride (Sigma-Aldrich), followed by thorough washing with PBS. Untreated (non-AGEd) wells were treated the same, with the exception of PBS being substituted for glycolaldehyde. To confirm the presence of AGEs in our experimental model, ECM was also seeded into black 96-well plates and treated as described above. Maillard-type fluorescence (excitation 370 nm, emission 440 nm) was then measured using a fluorescent plate reader (BMG Labtech, Aylesbury, UK).

Human fetal RPE cells were trypsinized, and approximately 1.5×10^5 cells were seeded per Transwell or 2.5×10^5 per standard well. Although all hfRPE monolayers used were expected to be highly polarized, this could only be confirmed experimentally when cultured in Transwells. Therefore, to distinguish between these two culture methods, we refer to Transwell monolayers as "fully polarized" throughout the study. Cells were cultured in media containing 15% fetal calf serum (FCS) overnight and subsequently cultured in standard 5% growth media for the remainder of the experiments (up to 10 weeks). Most experiments were carried out on cells from donors 1 and 2, and in a few cases, donor 3 (stated in the figure legends). For each donor, at least three separate replicate wells were used for each experimental condition. When comparisons of cell numbers were required between treatment groups, quantification was carried out using an MTT assay kit (Life Technologies), with relative data expressed as optical density (OD) units.

Determination of Transepithelial Resistance (TER)

The TER across hfRPE monolayers in Transwells was measured with a volt-ohm meter within 10 minutes of removing the culture plate from the incubator. All wells were measured three times and averaged. The TER of a blank well was used as a baseline value and subtracted from all experimental measurements, and the final TER was calculated by multiplying by the effective growth area of the Transwell (1.12 cm^2).

Western Immunoblotting

At least two separate conditioned media samples were collected and analyzed for each experimental condition, 48 to 72 hours after change of media. When using Transwells, the volume of the upper chamber was normalized to match that of the lower chamber using serum-free media. Unconditioned, full-serum growth media were previously analyzed by Western blot and produced no bands following probing with any of the antibodies used in this study (this ensured that all analyzed proteins were present in conditioned media as a result of hfRPE secretion). For conditioned media samples, normalization of protein content was carried out before analysis, as the effects of AGEs on secretion of potential loading controls was unknown. Protein content was determined using a Qubit fluorometer (Life Technologies), and protein was loaded equally by mass. Cell lysates were collected following addition of lysis/sample buffer[14] to wells.

Samples were resolved by SDS-PAGE, alongside a molecular weight marker (Thermo Scientific, Northumberland, UK), and Western blot analysis was then carried out as previously described.[10] An aliquot of the same random sample of conditioned media was loaded onto every gel as an internal control to normalize for differences between blots. Primary antibodies used were anti-cystatin C (Millipore, Watford, UK) diluted 1:2000, anti-pigment epithelium–derived factor (anti-PEDF) (Millipore) diluted 1:1000, and anti α-tubulin (Sigma-Aldrich) used at 1:10,000 dilution. Secondary antibodies used were horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse (Sigma-Aldrich). Membranes were prepared for chemiluminescence measurements using ECL Prime Western Blotting Detection Reagent (GE Healthcare, Little Chalfont, UK) and imaged using a ChemiDoc (Bio-Rad, Hertfordshire, UK). Densitometry of bands was carried out using ImageLab software (Bio-Rad). Final optical density values were calculated by normalizing all readings to that of the internal control on each blot (given an arbitrary value of 1), and in the case of cell lysates, by also normalizing to the loading control (α-tubulin).

ELISA for Cystatin C

Conditioned media samples were collected from hfRPE monolayers grown in standard 24-well culture plates at varying

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time points after replacement (24, 48, and 72 hours). Following a 1:4 dilution, cystatin C concentration was determined using a Quantikine human cystatin C ELISA Kit (R&D Systems, Abingdon, UK), following the manufacturer's protocol. Values obtained (nanograms per milliliter) were multiplied by four (dilution factor) to obtain final concentrations.

Real-Time Quantitative PCR (qPCR)

RNA was isolated from cells using the RNeasy Plus Mini-Kit (Qiagen, West Sussex, UK). Complementary DNA was synthesized from 0.3 μg RNA using an RT^2 First Strand Synthesis Kit (Qiagen). Quantitative PCR was performed using the MESA BLUE qPCR Mastermix Plus Kit for SYBR assay (Low ROX; Eurogentec, Southampton, UK) using a modified version of a previous protocol.[31] The primer set used for cystatin C was 5′-GCCGAACCACGTGTACCAAG-3′ and 5′-AGGTGGATTTCGA CAAGGTCATT-3′. Final values are expressed relative to a calibrator sample assigned an arbitrary value of 1 and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH; primer set: 5′-AACAGCCTCAAGATCATCAG-3′ and 5′-TGAGTCCTTCCACGATACC-3′) using the efficiency-corrected ΔΔCt method. Reactions were run on a Stratagene MX3000P qPCR System (Stratagene, La Jolla, CA), and three technical replicates were used for each cDNA sample. The specificity of amplification reactions was confirmed by melt-curve analysis and agarose gel electrophoresis.

Donor Tissue Preparation for Immunohistochemistry

The local research ethics committee granted ethical approval for the study, and the research adhered to the tenets of the Declaration of Helsinki. Human eyes were obtained from the local eye bank (Royal Liverpool University Hospital, Liverpool, UK). Thirty-five human eyes, each obtained from a different donor with no history of ocular disease and with an age range of 17 to 95 years were used. Eyes were identified only by their specimen number throughout the study, ensuring that they remained anonymized and age masked. Unmasking was carried out following data collection, to allow for final analysis. Tissue sections were prepared as previously described.[32]

Immunohistochemical Analysis

Sections were processed as previously described,[32] with the addition of a protein block with 20% goat serum (Dako, Ely, UK). Incubation with 5 μg/mL of rabbit anti-cystatin C (Millipore) was then carried out. Negative control sections had primary antibody omitted (incubated in 1% goat serum). Sections were then incubated with Dako EnVision anti-rabbit labeled polymer-HRP conjugate. Visualization was achieved using Dako 3-amino-9-ethylcarbazole substrate. Sections were then mounted in Vectormount AQ aqueous mounting media (Vector Laboratories, Peterborough, UK). In order to ensure that variability of staining was reduced, the conditions for the immunohistochemical staining were first optimized and repeated on a subset of specimens, which were then included and compared in each run alongside all specimens.

The RPE cells were imaged using a light microscope with a digital camera, as previously described.[32] Images were taken of the posterior pole segment (corresponding to the macula) and compared with the peripheral RPE of each specimen. Analysis was carried out using ImageJ software (National Institutes of Health, Bethesda, MD). Images were inverted (white = grayscale value of 0, and black = grayscale value of 255), and the mean gray value for each area was measured. For each image, the average of the three mean gray-value measurements was taken, and the mean gray value of the negative control most closely resembling the background conditions of the sample was deducted. Posterior pole and periphery segments were analyzed separately. For this study, posterior RPE cells were defined as those that lay temporal to the optic nerve and within the area of the retinal vascular arcades; peripheral RPE cells were defined as those that lay anterior to the equator of the globe. Periphery measurements of opposite sides of each globe were averaged.

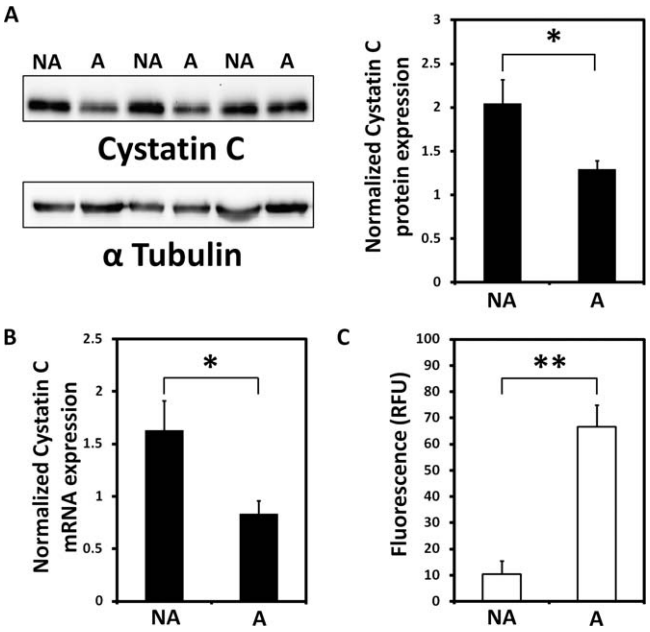

Figure 1. Cystatin C expression in hfRPE cells on AGEd ECM. (**A**) Representative Western blot shows cystatin C and α-tubulin expression of hfRPE cells (1 donor displayed) from three separate untreated-(non-AGEd) and three separate AGE-treated (AGEd) wells. Graph shows the average normalized cystatin C expression (arbitrary units) + SEM for all samples tested (donors 1 and 2); for each condition, $n = 6$. (**B**) Cystatin C mRNA expression in hfRPE cells as determined by qPCR analysis. Graph shows average normalized expression (arbitrary units) + SEM for all samples tested (donors 1 and 2); for each condition, $n = 6$. (**C**) Advanced glycation end-product–specific fluorescence on both untreated and treated ECM samples; for each condition, $n = 4$. Independent *t*-test; $^*P < 0.05$, $^{**}P < 0.01$. NA, non AGEd; A, AGEd.

Statistical Analysis

All statistical analysis was carried out using SPSS for Windows (Version 20; IBM, Portsmouth, UK). A *P* value of less than 0.05 was considered to be statistically significant.

Results

AGE Accumulation on the Basal ECM Impacts on Cystatin C Expression

To determine the effects of aging on the expression and secretion of cystatin C in the RPE, an in vitro system was used with the ability to mimic one of the most common biochemical changes associated with aging and age-related disease: the accumulation of AGEs on long-lived protein structures.

Human fetal RPE cells were cultured for 2 weeks on either untreated ECM (non-AGEd), or ECM pretreated to induce the accumulation of AGEs (AGEd). Whole cell lysates from these cells were then used to analyze cystatin C expression by Western blot and qPCR (Fig. 1). This experiment revealed

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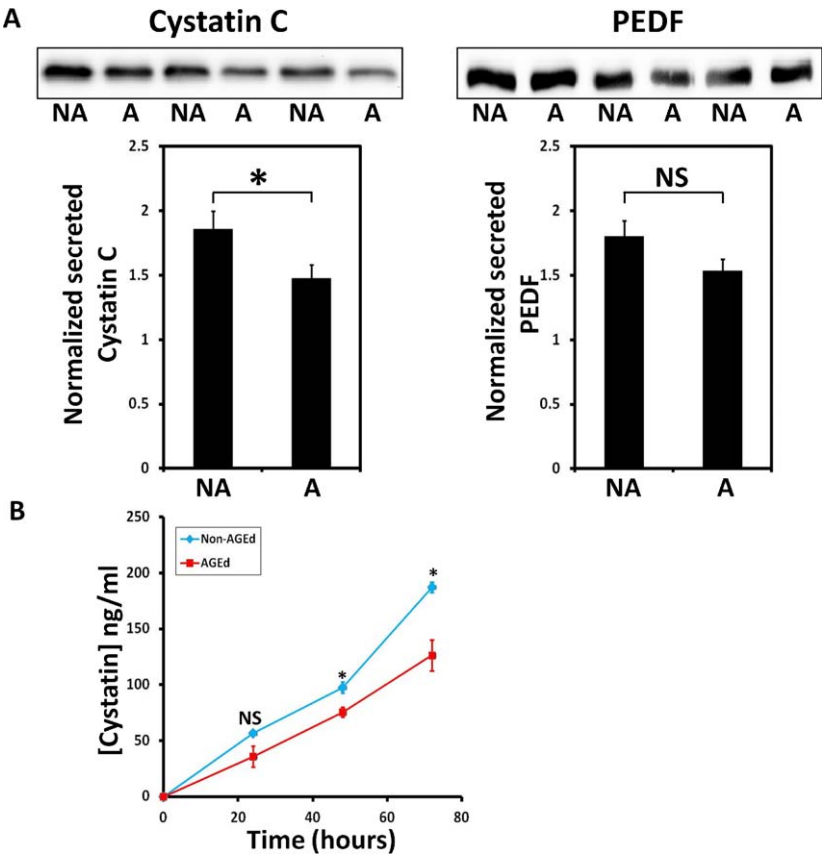

Figure 2. Cystatin C secretion from hfRPE monolayers cultured on AGEd ECM. (**A**) Secreted cystatin C and PEDF levels in hfRPE conditioned media (one donor displayed) from three separate untreated- (non-AGEd) and three separate AGE-treated (AGEd) wells. All blots are representative of observed secretion patterns. Graphs show average normalized cystatin C and PEDF secretion (arbitrary units) + SEM for all samples tested (donors 1 and 2); for each condition, $n = 12$. (**B**) Concentration of cystatin C over time in hfRPE conditioned media from three separate untreated- (non-AGEd) and three separate AGE-treated (AGEd) wells (determined by ELISA analysis). Concentration is displayed as nanograms per milliliter ± SEM for all samples tested (donor 3); for each condition, at each time point, $n = 3$. Independent *t*-test, $^*P < 0.05$. NS, not significant.

cystatin C expression was decreased as a result of AGE-accumulation on the basal substratal ECM on which RPE cells were grown. A similar decrease was observed at both the protein (Fig. 1A) and RNA levels (Fig. 1B). Expression levels of genes used for normalization (α-tubulin for protein analysis and GAPDH for mRNA analysis) were not significantly affected by AGE accumulation (values used to normalize cystatin C data, not shown separately). An increase in AGE-specific fluorescence on ECM following glycolaldehyde treatment confirmed the presence of AGEs in our experimental model (Fig. 1C).

AGEd Basal ECM Decreases Cystatin C Secretion by RPE

The next step was to determine if the observed reduction in cystatin C protein expression resulted in a corresponding decrease in cystatin C secretion from the RPE monolayer. Conditioned media collected from the cells were analyzed by immunoblotting for cystatin C alongside PEDF as a control, known to also be secreted at high levels from hfRPE cells.[30,33] Secretion of cystatin C was significantly decreased following culture on AGEd ECM for 2 weeks compared with non-AGEd (Fig. 2A), while PEDF was secreted at similar levels from cells cultured on both AGEd and non-AGEd ECM. ELISA analysis revealed that total secretion of cystatin C from hfRPE monolayers averaged approximately 57 ng/mL/24 h (Fig. 2B). Secretion levels were significantly reduced following culture on AGEd ECM at both the 48- and 72-hour time points.

Polarized Secretion of Cystatin C From hfRPE Cells on Non-AGEd and AGEd ECM

To analyze polarized secretion of cystatin C, as well as potential effects on this pattern caused by the presence of AGEs, hfRPE cells were cultured on non-AGEd and AGEd ECM in Transwell inserts (fully polarized). This allowed cells to establish distinct apical and basolateral secretion patterns. Cells were monitored over an extended period of time (up to 10 weeks). Conditioned media were collected from upper and lower chambers of Transwells at different points throughout the 10-week time course. After 4 weeks, non-AGEd monolayers reached a TER of approximately 250Ω·cm^2 and showed equal secretion of cystatin C into both the upper (apical) and lower (basal) chambers (Fig. 3A). Pigment epithelium–derived factor secretion was also nonpolarized at this stage. However, AGEd monolayers had not attained the minimum TER of 200Ω·cm^2, so they were not analyzed for polarized secretion at this stage.

The AGEd monolayers reached the threshold TER of 200Ω·cm^2 after 8 to 0 weeks post seeding, so direct comparisons could now be made of non-AGEd and AGEd polarized secretion. At this stage, cystatin C was secreted significantly more abundantly into the basal chamber of non-AGEd monolayers, whereas PEDF was secreted preferentially into the apical chamber in line with previous reports[30,33] (Fig. 3B). In AGEd monolayers, however, secretory polarity of both cystatin C and PEDF was not present, with both proteins being secreted equally into apical and basal chambers (Fig. 3C).

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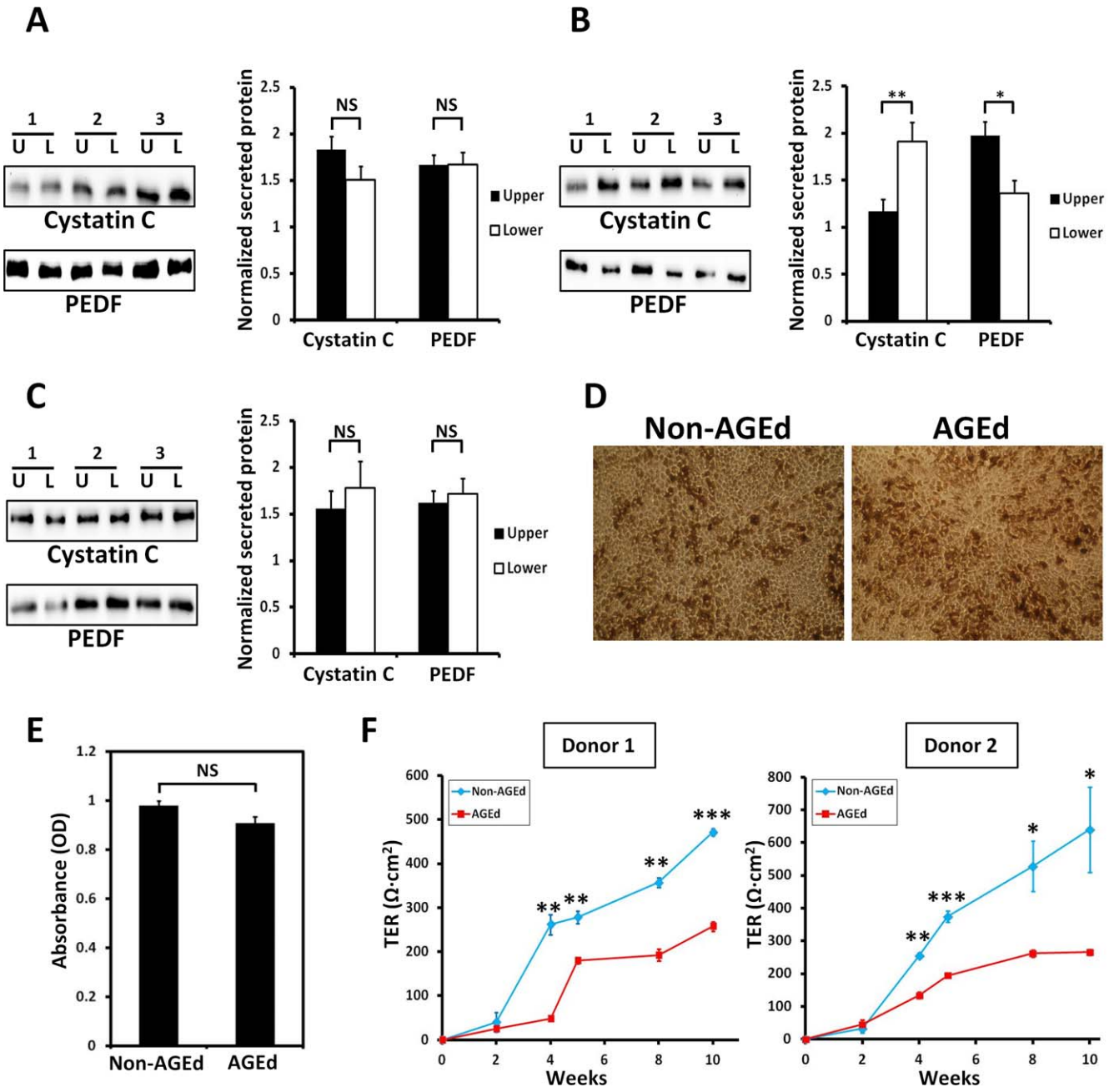

Figure 3. Polarized secretion of cystatin C by fully polarized hfRPE monolayers and effects of the presence of AGEs. Secreted cystatin C and PEDF levels in conditioned media collected from the upper (U) and lower (L) chamber of three separate hfRPE monolayers following (**A**) 4 weeks of culture in non-AGEd wells, (**B**) 8 to 10 weeks culture in non-AGEd wells, or (**C**) 8 to 10 weeks culture in AGEd wells. All blots show results from three separate wells (one donor displayed) and are representative of observed secretion patterns from all samples tested. Graphs show average normalized cystatin C and PEDF secretion (arbitrary units) + SEM for all samples tested (donors 1 and 2); for each condition, $n = 12$ (paired t-test, $^{*}P < 0.05$, $^{**}P < 0.01$). (**D**) Images show the typical morphology of hfRPE cells grown for 10 weeks on either untreated- (non-AGEd) or AGE-treated (AGEd) Transwells. (**E**) Comparison of cell number of hfRPE monolayers (non-AGEd versus AGEd) 2 weeks after seeding. Measurements expressed as OD units (MTT assay) + SEM for all samples tested (donor 3); for each condition, $n = 3$. (**F**) Transepithelial resistance measurements across hfRPE monolayers over 10 weeks. Graphs show measurements ± SEM from both donors studied after culture on untreated- (non-AGEd) or AGE-treated (AGEd) Transwells; for each condition, $n = 3$. Independent t-test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0001$. NS, not significant.

Morphological and physiological (TER) characteristics of the hfRPE were monitored to determine AGE-related changes. In the majority of cases, hfRPE cells were able to form typical monolayers on both non-AGEd and AGEd ECM consisting of a tightly packed "cobblestone" arrangement of pigmented cells (Fig. 3D). In a small number of cases, cells seeded onto AGEd ECM initially adhered less efficiently, presenting holes in the monolayer within the first week. These holes usually closed within a few days, resulting in a monolayer similar to those produced on non-AGEd ECM (monolayers in which holes remained beyond 1 week post seeding were not used for any subsequent polarized secretion analysis). No differences in cell number were observed between non-AGEd and AGEd cultures (Fig. 3E) following 2 weeks of culture (the point at which cell monolayers have reached complete confluence and attained a cobblestone pattern).

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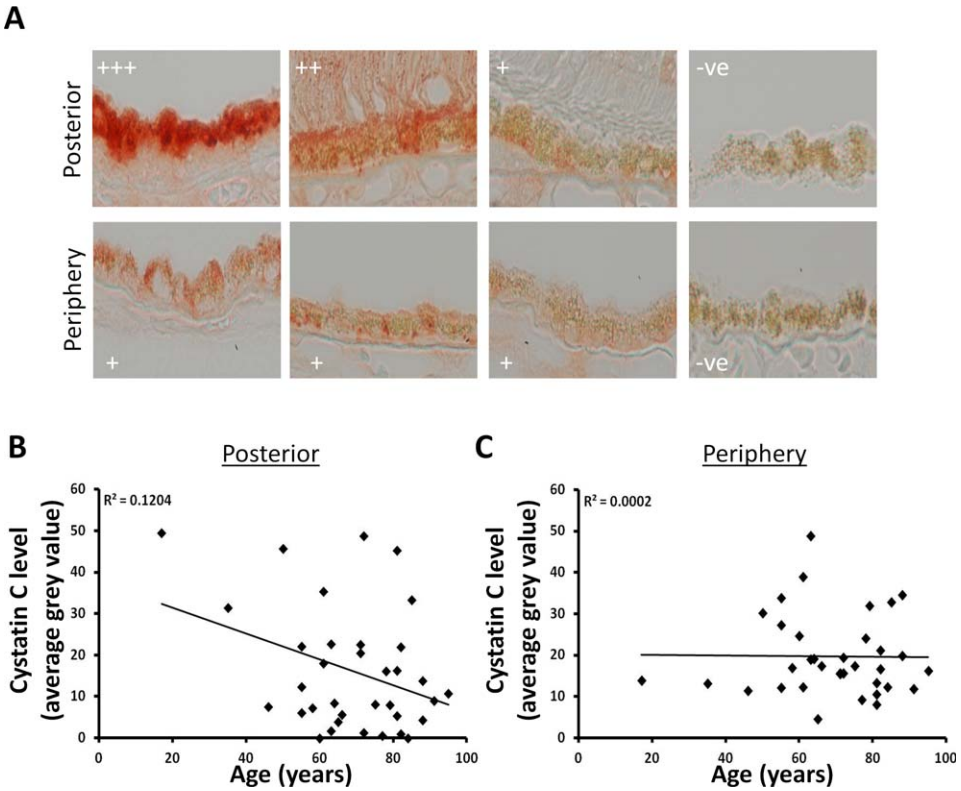

FIGURE 4. Cystatin C protein expression/accumulation in the RPE in situ. (**A**) Grading of cystatin C immunoreactivity in sections from posterior and peripheral RPE segments (positive staining displayed in grades of increasing intensity from + to +++; -ve indicates primary antibody omitted). (**B**, **C**) Relationship between cystatin C protein expression/accumulation with age. Cystatin C protein level was plotted against the age of each individual donor, in both posterior (**B**) and peripheral (**C**) RPE segments. The line of regression was significant for posterior samples ($P < 0.05$, $R^2 = 0.1204$, $n = 35$), but not peripheral ($R^2 = 0.0002$, $n = 35$).

A sharp and constant increase in TER was observed in non-AGEd monolayers (Fig. 3F), typically reaching approximately 200 to 250Ω·cm^2 within 28 days as previously reported.[30,33] Transepithelial resistance increased at a much slower rate for AGEd monolayers, only reaching 200Ω·cm^2 at around 8 (donor 2) to 10 weeks (donor 1) (Fig. 3F). The consistently increasing TER readings taken for all conditions indicate the presence of a functional, intact monolayer. All cultures analyzed maintained a minimum TER of 200Ω·cm^2 at the final point of analysis.

Cystatin C Relative Protein Level Decreases With Age in the RPE Underlying the Macula Region

To analyze the relationship between cystatin C expression/accumulation and age in situ, RPE-containing sections derived from the eyes of 35 human donors were tested for cystatin C immunoreactivity. The protein was present in all samples tested, with varying grades of intensity dependent upon anatomic location (Fig. 4). Intense staining was observed more frequently in posterior RPE segments (location of macula) than in the periphery (Fig. 4A). Analysis of staining intensity in relation to age revealed a negative correlation between cystatin C level and increasing age in the posterior (macula) RPE segment, but not in the peripheral segment (Figs. 4B, 4C).

DISCUSSION

We demonstrated a marked decrease in cystatin C expression in hfRPE cells cultured on AGEd ECM. This was apparent at the transcriptional level, leading to decreased protein expression, and ultimately, reduced secretion. Furthermore, the physiologically relevant basal polarity of its secretion was lost following prolonged exposure of cells to AGEs. Previously, the association between cystatin C and AMD risk was attributed to the malfunction in intracellular targeting, and subsequent decreased secretion of the variant B homologue.[14] Here, we demonstrated that a physiological change associated with the aging process can also affect secretion of wild-type cystatin C.

The in vitro model used in our study was adapted from published protocols[23,28,29,34] and utilized the previously documented accumulation of AGEs on long-lived protein structures; in this case the substratal ECM on which cells were cultured. A necessary adaptation was in the use of a slightly different ECM in order to provide optimal growth conditions for primary hfRPE cells, which were used to ensure confluent monolayers with physiological characteristics of native human RPE.[30] The components of the ECM used (predominantly collagen IV and laminin) were however almost identical to ECM mimics used in other RPE studies[23,28,29] and provided both an accurate representation of the BrM and a suitable substrate for glycation reactions.[34] Human fetal RPE cells formed consistent and robust monolayers displaying the characteristic cobblestone morphology and maintained high levels of pigmentation. The use of primary hfRPE cells, as opposed to transformed cell lines, contributed to a physiology similar to that of native RPE,[30,33] resulting in a suitable model for analyzing secretion/polarity.

The present study also ascertained for the first time that in situ the abundance of wild-type cystatin C is reduced in the macula region of the RPE/choroid as a result of increasing age. Varying demands placed on RPE cells in different regions of the retina can result in altered gene expression profiles,[35] particularly for genes associated with the ECM. It is conceiv-

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able that cystatin C expression also varies depending on topographic location, as evidenced in the present study, where relative protein levels were higher in the macula across all age groups compared with peripheral regions. An age-related decrease in cystatin C in such regions of high demand could therefore modify the local proteolytic balance. Furthermore, the decrease would be greatly accelerated in the variant B expression scenario, thus explaining the increased risk posed by the respective allele for AMD pathogenesis. Notably, the reduced level and/or secretion of cystatin C, whether age-related or consequential to variant B expression, was suggested as a molecular factor responsible for increased neuronal vulnerability and risk of neurodegenerative diseases, including Alzheimer's disease.[13,36–39]

Considering its known enzyme targets, it is likely that cystatin C is involved in proteolytic events that regulate either maintenance of the BrM or cell-signaling processes on the basolateral side of RPE. Reduced protein level in this region as a result of decreased secretion could contribute to AMD-associated changes, such as disruption in the stability and function of the BrM,[40–42] and increased permeability of RPE.[43,44] There is also evidence that cystatin C can interact with complement component 4,[45] and by doing so, may inhibit the complement cascade. It is therefore possible that reduced levels of cystatin C secretion could also contribute to the increased complement activity often associated with AMD pathogenesis.[46]

Advanced glycation end product–induced gene expression changes in the RPE were previously documented both at the transcriptional[23,29] and protein levels,[28] yet how AGEs alter cystatin C expression remains to be determined. The cystatin C promoter shares many similarities to those of housekeeping genes, suggesting constitutive expression[47]; however, certain transcription factors, such as interferon regulatory factor 8 can bind to the promoter and regulate its activity.[48] Transcriptional regulation can also be achieved following treatment with dexamethasone[49] and TGFβ.[50] Cystatin C is ubiquitously expressed in human tissues, yet its abundance can vary greatly, even among similar cell types.[51] This suggests constitutive expression, subject to cell-specific regulation via particular transcription factors.

Multiple mechanisms exist by which AGEs can alter gene expression. The direct mechanism is via activation of the receptor for advanced glycation end-products (RAGE). Retinal epithelial cells express RAGE, and its activation can lead to the alteration of cellular processes such as protein secretion.[52] RAGE can modulate a multitude of downstream pathways including mitogen-activated protein (MAP) kinase signaling via p21 (ras),[53] cdc42 and Rac-1,[54] extracellular signal-related kinase, c-jun N-terminal kinase, and p38.[55] RAGE can also increase nuclear factor kappa B (NF-κB) transcriptional activity.[56] Alternatively, AGEs can affect gene expression via indirect mechanisms. These can include an increase in oxidative stress associated with AGE accumulation,[57–59] and the subsequent alteration of multiple cellular functions.

Our results suggest that the gradual exposure of RPE cells to AGEs may impair their ability to maintain a highly polarized state and secretion program, which in turn could, in time, affect the overall transport and, potentially, barrier function of the monolayer. This is underscored by the alteration in the directional secretion of not just cystatin C but also PEDF, indicating a wider effect on the cells. Indeed, it was demonstrated that attainment of a fully polarized conformation in culture is enough to augment overall protein secretion by hfRPE,[60] highlighting the importance of this state for correct functional secretion patterns. Furthermore, in a study comparing the cell line ARPE19 (suggested by the authors to more closely mimic an aged phenotype) to hfRPE, a decrease in total VEGF secretion was observed, and also alterations in its polarized secretion were noted.[61] These findings pointed out the potential of the aging process to alter the polarized state of cells. In alveolar type II cells, varying components of the substratal ECM can affect intracellular organization, cell polarity, and TER measurements.[62] Advanced glycation end product–induced changes in the BrM could have similar consequences on RPE cells. ARPE19 cells cultured on an AGE-treated BrM mimic for 4 weeks displayed marked ultrastructural differences to those cultured on untreated ECM.[23] Perhaps the most relevant of these changes, in terms of maintained barrier function, was in the apparent reduction in tight-junction formation in AGE-treated cells. Studies in retinal endothelial cells demonstrated that short-term exposure to AGEs can increase permeability across the monolayer, possibly by affecting tight junctions.[63] A similar short-term study carried out in ARPE19 cells was also able to show an AGE-dependent decrease in the barrier function of the monolayer, resulting in decreased TER measurements.[64] Our study represents the first analysis of the long-term effects of AGEs on well-polarized RPE function, applied to a specific gene-expression product.

The ability to fully replicate the physiological process of AGE accumulation in culture has limitations. It is not possible to gradually bring AGEs into an ECM already supporting live cells, as occurs in vivo. This limitation is, however, partly countered by the deposition of new non-AGEd ECM in vitro[4,65] during the formation of fully confluent monolayers, potentially modulating AGE exposure in culture conditions. In situ, AGE accumulation increases with age, resulting in a long-term insult that would likely affect the ability of RPE to retain a polarized state over time, and to maintain a functional, impermeable barrier to diffusion.

We conclude that a common pathological change associated with the aging process, the accumulation of AGEs, can alter both the expression of cystatin C, and the polarity of its secretion. It is possible that each of these phenomena is the result of different AGE-induced molecular changes, which may, or may not, occur simultaneously in situ. It is clear, however, that even alone, each of these changes can lead to the same scenario, a significant decrease in cystatin C protein level at its sites of action. Coupled with the previously established knowledge regarding decreased secretion of the AMD-associated variant B,[14] these findings are consistent with the involvement of cystatin C in AMD pathophysiology. The observed reduction in the extracellular cystatin C protein level is likely to contribute to reduced control over proteolytic homeostasis in the retina/choroid, which in turn may contribute to the well-documented biological changes that occur with age at this site.[66] The potential broader effects exerted by AGEs on RPE polarity and barrier function are another important finding. Such AGE-induced changes have the potential to alter the localization of a multitude of factors and may contribute to disease pathogenesis in a similar way to cystatin C mis-localization.

Acknowledgments

The authors thank Sheldon Miller, National Eye Institute, National Institutes of Health, for the advice and support regarding use and provision of the human fetal donor RPE.

Supported by a grant from R & D Royal Wolverhampton NHS Trust (LP). Work in Luminita Paraoan's lab is supported by AgeUK.

Disclosure: **P. Kay**, None; **Y.C. Yang**, None; **P. Hiscott**, None; **D. Gray**, None; **A. Maminishkis**, None; **L. Paraoan**, None

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