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Clusterin protein diversity in the primate eye

Paul Wong,[1] Bruce A. Pfeffer,[2] Steven L. Bernstein,[3] Michelle L. Chambers,[1]Gerald J. Chader,[4] Zahra F. Zakeri,[5] Yan-Q Wu,[6] Mark R. Wilson,[7] S. Patricia Becerra[6]

[1]Departments of Biological Sciences, Ophthalmology, and Medical Genetics, University of Alberta, Edmonton, Alberta, Canada; [2]Bausch and Lomb Pharmaceuticals, Pearl River, NY; [3]Department of Ophthalmology, University of Maryland, Baltimore, MD; [4]Foundation Fighting Blindness Inc., Hunt Valley, MD; [5]Queen's College, CUNY, New York, NY; [6]Laboratory of Retinal Cell and Molecular Biology, National Eye Institute, National Institutes of Health, Bethesda, MD; [7]Department of Biological Sciences, University of Wollongong, Wollongong, NSW, Australia

Purpose: The clusterin gene encodes a multi-functional protein that has been identified in different tissues, including a number of different eye tissues, primarily in the mouse and to a much lesser extent in humans. Clusterin has been implicated in a number of cellular processes such as lipid transport, membrane integrity, apoptosis, and neurodegeneration, all of which could be important to the biology of the eye. In the current communication, we provide data that confirms the expression of clusterin in a number of different human eye tissues and establishes the expression profile of this gene in monkey derived eye tissues. The issue that we sought to examine is whether a broad profile of clusterin expression in the eye is consistent in primates (monkey and human).

Methods: The majority of our study was done using monkey eye tissues. Where possible, we have used human tissues in order to confirm published findings. Northern and western analysis was performed using tissues derived from monkey eyes. In situ hybridization and immunochemistry were carried out on human eye sections.

Results: Clusterin mRNA is expressed in primate lens, cornea, limbus, sclera, orbital muscle, ciliary body, retina, RPE/choroid, and RPE cells in culture. Western analysis revealed that two major groups of clusterin exist in the eye, a high molecular weight group (>100 kDa) and a second group consisting of at least five clusterin species that are all approximately 80 kDa. Analysis of conditioned media from RPE cells cultured on permeable supports suggests that different forms of clusterin display alternative patterns of secretion.

Conclusions: Clusterin is expressed in a broad range of eye tissues in both human and monkey, suggesting that this is a characteristic feature in primates. We demonstrate for the first time that a diverse number of clusterin isoforms were observed in monkey eye tissues by western analysis. Meanwhile, the molecular size of clusterin mRNA detected in the array of tissues are identical in size, suggesting that the nature of the diversity in clusterin forms is due to post-translational modifications. In addition, new insights were made in defining clusterin expression in ciliary body, cornea, and the retinal pigment epithelium.

Clusterin is widely expressed and highly evolutionarily conserved [1-4]. The gene encoding clusterin was initially cloned as TRPM-2 [5-8], although it is also known by other names, including ApoJ and SGP-2 [1-4]. In many tissues, clusterin is expressed as a secreted glycoprotein that possesses potential amphipathic helical regions that may allow it to bind to hydrophobic molecules, as well as heparin binding domains that may mediate interactions with cell membranes and extracellular matrices [9]. The current understanding of clusterin biogenesis is that clusterin is initially synthesized as a 50 kDa holoprotein that is processed into a high-mannose-containing intermediate form of 58-65 kDa and subsequently modified into a complex carbohydrate form of 70-80 kDa [10,11]. Prior to secretion, the protein is typically cleaved to generate an α- and a β-subunit that in turn forms a dimer complex. The relative size of the protein in normal tissues is similar among different species [9,12-14]. Clusterin may play a role in a wide array of processes, including lipid transport, regulation of the complement cascade, active cell death, and membrane recycling [1-4]. Recently it has been suggested that the primary function of clusterin is the cytoprotection of cellular membranes or as a chaperonine [15,16]. All of these putative roles may be relevant to the normal function of clusterin in the eye.

In the human eye, clusterin mRNA and clusterin protein have been observed in aqueous fluid, ciliary body, cornea, retina, and vitreous fluid [8,17-21]. These observations were made largely on the basis of immunohistochemical and in situ analysis. In the present study we demonstrate for the first time, by western analysis, that there are a number of different isoforms of clusterin protein present in different normal ocular tissues, even though only a single transcript is detected after northern analysis. In addition, we confirmed the broad profile of clusterin expression in tissues of the human eye and demonstrated that this broad profile of expression is also present in monkey ocular tissues.

Correspondence to: Dr. Paul Wong, Department of Biological Sciences, University of Alberta, G504 Biological Sciences CTR Edmonton, Alberta, Canada, T6G 2E9; Phone: (780) 492-3950; FAX: (780) 492-9234; email: pwwong@gpu.srv.ualberta.ca

Dr. Wu is now at the Department of Ophthalmology, First Affiliated Hospital, Suzhou Medical College, Suzhou, People's Republic of China.

METHODS

Tissues: Eyes from juvenile (under three years of age) rhesus monkeys, *Macaca mulatta*, were kindly provided by Mr. J. Cogan (Bureau of Biologics, Bethesda, MD). Animals were cared for according to the "Principles of laboratory animal care" (NIH publication No.86-23, revised 1985). Eyes were enucleated within 3 h postmortem and kept on ice. Time from euthanasia until dissection was 2 h. Following penetration of the pars plana with a needle knife, the anterior segment was dissected from the sclera using corneoscleral scissors, 2 mm from the limbus. The lens was removed, and the ciliary body dissected and frozen on dry ice. The iris was dissected and treated similarly. The boundaries of the trabecular meshwork (TM) were identified, and the TM removed using sharp dissection with a sharp blade. The corneal-scleral limbus tissue was dissected 1 mm from clear cornea, and all tissues were frozen on dry ice. Clear vitreous was dissected from the posterior chamber using syringe suction and sharp tipped Wescott scissors. The neuroretina/retinal pigment epithelium (RPE) oxygen status was determined by the association of the two tissues. Fresh globes have a strong association of RPE and neuroretina, requiring careful neuroretinal dissection using subretinal air injection. Following removal of the neuroretina, the choroid/RPE complex was dissected from the sclera. Both choroid/RPE and sclera were fast frozen on dry ice. Samples used for the study are trabecular meshwork (TM), lens, cornea, limbus, sclera, orbital muscle, iris, ciliary body, RPE/choroid, and retina tissue. All were dissected on ice, and then frozen on dry ice and stored at -80 °C. Samples of juvenile monkey vitreous were kindly provided by Dr. Paul Russell (NEI, NIH, Bethesda, MD).

RPE cell culture: Confluent cultures of first passage rhesus monkey RPE cells were utilized to generate conditioned media samples (CM). The methodology used for establishing RPE cultures has been described in detail [22]. Cells were maintained as stable monolayers in 12-well clusters (Costar, Cambridge, MA) for two weeks, in medium (described below) designed to optimize the expression of a variety of biochemical and physiological markers characteristic of the differentiated tissue in vivo [22]. Confluent wells contained 6 x 10^5 cells each, as calculated from hemacytometer counting of parallel cultures. For production of conditioned media, RPE cells were maintained in a protein-free defined medium that was modified from that described by Pfeffer [22] in the following ways: (1) Serum, bovine retinal extract, insulin, and transferrin were omitted; (2) Fatty acid bovine serum albumin complex was replaced by Chemically Defined Lipid Concentrate (GIBCO-BRL, Gaithersberg, MD). After an eight day conditioning period, the pH of the medium remained physiological and cell morphology was unchanged.

In order to investigate the polarized release of clusterin, monolayers of RPE cells were established on 9 mm diameter cell culture inserts containing 0.4 µm pore size polyethylene terephthalate membranes (Falcon, Franklin Lakes, NJ). Cultures were monitored visually under an inverted microscope to assess cell proliferation and morphology at confluence. A stable, confluent monolayer was attained by 8 days and the routine culture medium in both chambers was replaced with defined medium lacking serum and retinal extract. Thereafter, the harvesting of medium from apical (0.5 ml) and basal (1.2 ml) compartments, and its replenishment, was repeated in cycles of alternating 4 and 3 days for approximately two months. Conditioned media samples were frozen and stored at -70 °C. Similar preparations in our hands have been shown, with the use of a Millicell-Electrical Resistance System (Millipore, Bedford, MA), to develop a transepithelial resistance in the range of 30 to 50 ohm-cm^2.

Western analysis: Tissues were homogenized in lysis buffer (25 mM Tris-Cl pH 8.0, 200 mM NaCl, 20 mM glycerol, 1% Triton X-100, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 10 µg/ml aprotinin). Homogenates were centrifuged and the supernatant containing soluble protein was isolated. Soluble fractions, as well as aqueous, vitreous, and conditioned media from monkey RPE cultures were subjected to SDS-PAGE on 10% acrylamide gels [23]. Proteins were transblotted from polyacrylamide gels onto nitrocellulose by the method of Towbin et al. [24]. Blots were blocked in 1% BSA in Tris-buffered saline (20 mM Tris, 0.15 M NaCl, pH 7.5) containing 0.05% Tween-20 (TBST) for 2 h and incubated with G7, a monoclonal antibody raised against human clusterin [25], at a 1:1 dilution for 16 h at 4 °C. The blot was then treated with biotinylated anti-mouse IgG (Vector Laboratories, Burlington, CA), followed by an avidin-biotinylated horseradish peroxidase (HRP) complex (Vectastain Elite ABC kit, Vector Laboratories). Finally, the HRP complex was detected using an HRP color development kit (Bio-Rad, Rockville Centre, NY), as previously described [26]. The clusterin specificity of the G7 antibody has been previously demonstrated [27,28]; it specifically detects clusterin in its non-reduced conformation.

In Situ hybridization and immunohistochemistry: Human eyes were obtained from National Disease Research Interchange (Philadelphia, PA) and fixed overnight in 4% paraformaldehyde in PBS. Eyes were then placed in 20% sucrose in PBS overnight, embedded in Optimum Cutting Temperature OCT Tissue-Tek; (Miles Inc., Elkhart, IN) and frozen in isopentane-liquid N_2. Frozen sections (6 µm) were cut, placed on poly-L-lysine coated slides, and stored at -70 °C prior to use.

For the in situ hybridization, the pG21-04 plasmid containing the clusterin/TRPM-2 cDNA [6] was used to generate both sense and anti-sense ^{35}S-UTP labeled RNA probes using standard protocols [29]. Sections were post-fixed for 5 min with 4% paraformaldehyde in PBS and treated with 10 mg/ml proteinase K in 1X standard saline citrate (SSC; 150 mM NaCl and 15 mM sodium citrate, pH 6.8) for 5 min; 2X SSC for 2 min; 1.31% triethanolamine, pH 8, for 2 min; 1.41% triethanolamine/0.25% acetic anhydride, pH 8, for 10 min; and 2X SSC for 10 min. Dehydrated slides were incubated in prehybridization buffer (50% formamide, 600 mM NaCl, 10 mM Tris, 1 mM ethylenediamine-tetraacetic acid (EDTA), 500 µg/ml Escherichia coli tRNA, 50 µg/µl salmon sperm DNA, 1X Denhardt's solution, and 0.5% sodium dodecyl sulfate (SDS)). The sections were hybridized in the same buffer with the labeled probes for 16-18 h at 50 °C. The slides were washed

with 2X SSC for 2 min, followed by 25 µg/ml RNase A in RNase buffer (500 mM NaCl, 1 mM Tris, and 1 mM EDTA, pH 7.5) for 30 min at room temperature, RNAse buffer for 30 min at 50 °C; 2X SSC for 1 h at 50 °C. Sections were dehydrated in ethanol, air dried and dipped in photographic emulsion. The slides were exposed for 2 weeks and developed in Kodak D-19 (Eastman Kodak Co., Rochester, NY) developer for 4 min at 14 °C, rinsed in water for 20 s at 14 °C, and fixed in Kodafix fixer for 6 min at 14 °C. The slides were stained with hematoxylin-eosin and viewed using a dark field microscope. In all cases, use of the sense probe (the standard control) only resulted in the detection of minor number of background silver grains (data not shown). As a more exact internal control for the hybridization of the anti-sense probe, we have been measuring the density of grains in regions of the slide without tissue relative to the areas corresponding to different regions of the tissue in question.

For immunohistochemistry, tissue sections were rehydrated through serial graded solutions of 20%, 10%, and 5% sucrose in PBS for 5 min each, followed by two washes in PBS for 5 min. Sections were blocked with 3% goat serum in PBS for 20 min at 37 °C to prevent nonspecific binding prior to incubation overnight at 4 °C with G7 (1/10 dilution in PBS). The protein was localized by using horseradish-peroxidase-linked secondary antibody and diaminobenzidine to develop a brown chromophore at the site of antibody binding.

Northern analysis: A human clusterin cDNA probe was obtained directly from available plasmids [6,30]. The probe was radiolabeled with [α-^{32}P]dCTP by oligonucleotide labeling to a specific activity of not less than 10^8 cpm/µg. Total RNA from dissected tissues was isolated using RNAzol (Tel-Test, Inc., Friendswood, TX) and from cultured RPE cells by the method of Peppel and Baglioni [31]. Total RNA was electrophoresed in 1% agarose gels in a formaldehyde running buffer system [32]. Northern transfer onto Genescreen Plus nylon membrane (Dupont-NEN, Boston, MA) was carried out by passive blotting in 10X SSC (1.5 M sodium chloride, 0.15 M sodium citrate, pH 7). Blots were UV crosslinked and heat baked for 2 h at 80 °C under vacuum before use. Prehybridization, hybridization, blot washes and autoradiography were carried out as previously described [7]. Profiles of TRPM-2/clusterin mRNA steady state levels were verified by analysis of duplicate northern blots.

Image analysis: The intensity of bands on autoradiographs and negatives was quantified using the Kodak Digital Science 1D image analysis system (Eastman Kodak Co., Rochester, NY). In the case of northern blots, we accounted for possible differences in band intensities due to differences in RNA loading by using the relative intensity of the 18S ribosomal band in each sample to normalize the respective clusterin value [33]. Statistical analysis was performed using Quatro Pro Version 7.0 software (Corel, Ottawa, Canada).

RESULTS

Northern blot analysis: Human clusterin mRNA is 1674 nucleotides in length [30]. In the monkey tissues examined by northern analysis, the monkey orthologous transcript presents as a single band of approximately 1700 nucleotides in length. The clusterin gene is widely expressed in monkey eye tissues (Figure 1, Table 1) although expression is not ubiquitous, being clearly undetectable in the TM or orbital muscle. Highest relative clusterin mRNA levels in Figure 1 (left panel) were found

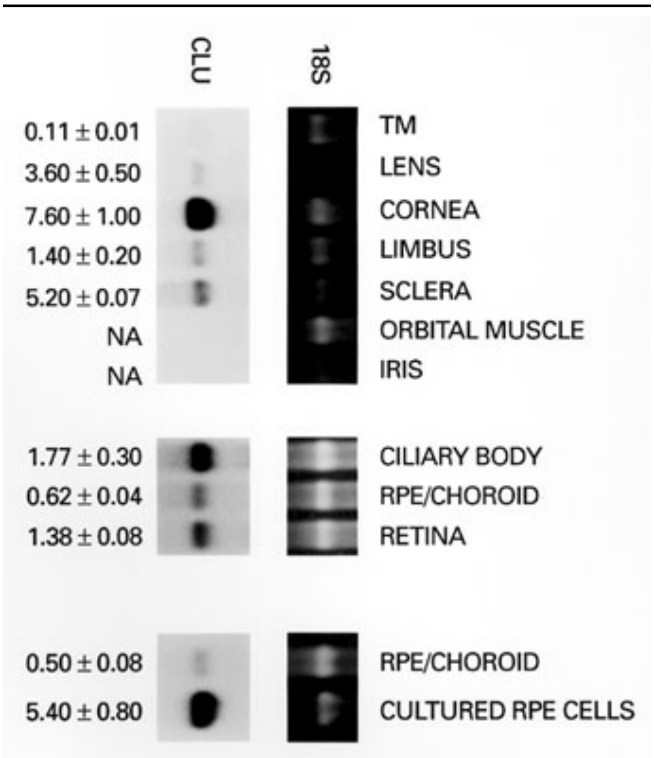


Figure 1. Clusterin mRNA levels in monkey ocular tissues. Representative northern blot analyses for clusterin expression are shown. Normalized mean clusterin values of multiple scans and standard deviations are shown below each lane. TM: trabecular mesh, RPE: retinal pigment epithelium.

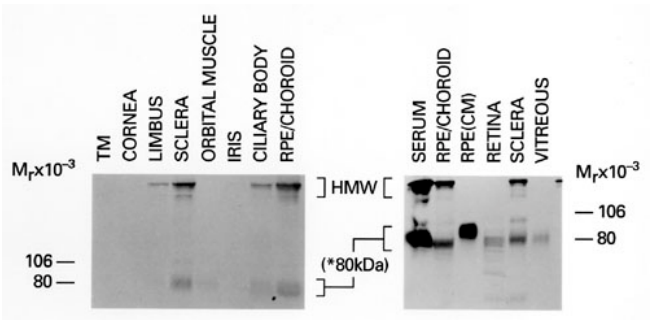


Figure 2. Clusterin protein distribution in monkey ocular tissues. Tissue lysates and tissue fluids were run on 10% SDS-PAGE. Clusterin was detected using a monoclonal antibody (G7) generated against human clusterin. Two subgroups of clusterin species were detected, a group of high molecular weight proteins (>100 kDa) and a second primary group of proteins of approximately 80 kDa in size (*80 kDa). TM: trabecular mesh; RPE: retinal pigment epithelium; CM: conditioned media.

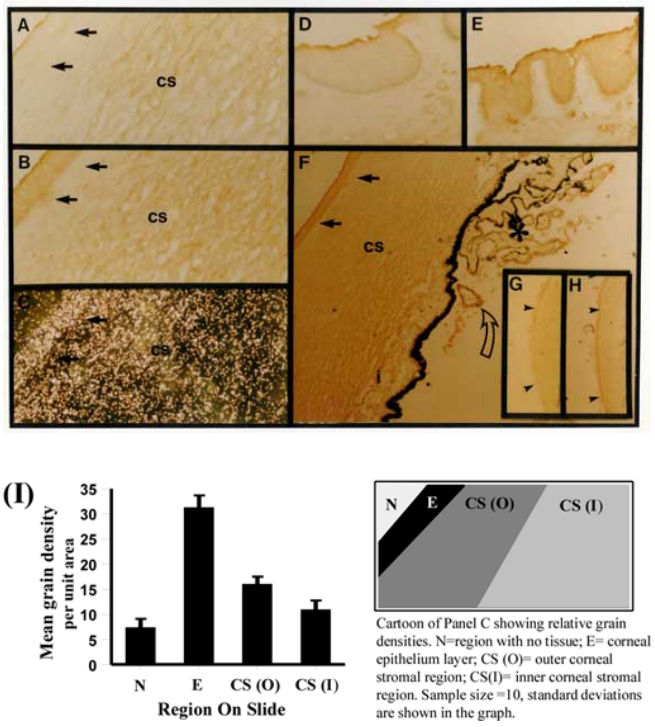
in cornea, lens and sclera. Analysis of ciliary body, RPE/choroid, and retina for clusterin mRNA shows that the message is present in all three tissues.

Western blot analysis: In monkey ocular tissues, two sets of clusterin protein bands are detected (Figure 2, Table 1). A prominent set of bands is found at approximately 80 kDa (*80 kDa species) and likely represents the intact clusterin protein molecule. The *80 kDa clusterin protein species is detected in orbital muscle, limbus (low levels), sclera, ciliary body, vitreous, retina, RPE, and conditioned media from RPE cells in culture. The *80 kDa clusterin protein species detected in vitreous, serum, and RPE conditioned media, which all represent secreted clusterin, are slightly larger than the *80 kDa clusterin found in tissue homogenates from RPE/choroid, retina, or sclera. Western analysis of monkey eye tissue samples performed in comparison to monkey serum showed that the clusterin protein in all the samples examined was distinct from serum clusterin (Figure 2), based on the electrophoretic mobility of the detected *80 kDa bands. A second high molecular weight (HMW, >100 kDa) group of clusterin protein variants is apparent in limbus, sclera, ciliary body, and RPE, and likely represents clusterin aggregates [12]. Both clusterin forms, as detected by the G7 antibody, are absent in TM and iris. Surprisingly, clusterin protein was not detected in cornea, which

TABLE 1. CLUSTERIN EXPRESSION IN THE PRIMATE EYE

Species	RNA/Protein (Method)	Expression Status	Source
Aqueous			
Human	Protein (W)	protein detected	[17]
Ciliary Body			
Human	Protein (IH)	protein detected	[17],*
Monkey	mRNA (N)	mRNA detected	*
Monkey	Protein (W)	protein detected (HMW, 80 kDa)	*
Cornea			
Monkey	mRNA (N)	mRNA detected	
Monkey	Protein (W)	protein not detected	
Human	mRNA (ISL)	mRNA detected	[19],*
Human	Protein (IH)	protein detected	[19],*
Cornea (epithelium)			
Human	mRNA (RT-PCR)	mRNA detected	[19]
Human	Protein (W)	protein detected	[19]
Eye lid/Skin			
Human	Protein (IH)	protein detected	*
Iris			
Monkey	mRNA (N)	mRNA not detected	*
Monkey	Protein (W)	protein not detected	*
Lens			
Monkey	mRNA (N)	mRNA detected	*
Human	Protein (IH)	protein detected	*
Limbus			
Monkey	mRNA (N)	mRNA detected	
Monkey	Protein (W)	protein detected (HMW, 80 kDa)	*
Orbital Muscle			
Monkey	mRNA (N)	mRNA not detected	*
Monkey	Protein (W)	protein detected (80 kDa)	*
Retina			
Human	mRNA (ISL)	mRNA detected	[17]
Human	mRNA (N)	mRNA detected	[18]
Human	protein (IH)	protein detected	[20]
Monkey	mRNA (N)	mRNA detected	*
Monkey	protein (W)	protein detected (80 kDa)	*
RPE			
Human	mRNA (ISL)	mRNA detected	[17]
RPE (cultured)			
Monkey	mRNA (N)	mRNA detected	*
Monkey	Protein (W)	protein detected (80 kDa)	*
RPE/choroid			
Monkey	mRNA (N)	mRNA detected	*
Monkey	Protein (W)	protein detected (HMW, 80 kDa)	*
Sclera			
Monkey	mRNA (N)	mRNA detected	*
Monkey	Protein (W)	protein detected (HMW, 80 kDa)	*
Trabecular Mesh			
Monkey	mRNA (N)	mRNA detected	*
Monkey	Protein (W)	protein not detected	*
Vitreous			
Human	Protein (W)	protein detected	[17]
Monkey	Protein (W)	protein detected (80 kDa)	*

Summary of reports of clusterin expression in the primate eye. An asterisk ("*") in the source column indicate that the finding is reported herein. This report includes new observations of clusterin expression in eyelid, iris, lens, limbus, orbital muscle, cultured RPE, sclera, and trabecular meshwork. (W = Western analysis, IH = Immunohistochemistry; NA = Not applicable; ISL= In situ localization; N = Northern analysis)



Cartoon of Panel C showing relative grain densities. N=region with no tissue; E= corneal epithelium layer; CS (O)= outer corneal stromal region; CS(I)= inner corneal stromal region. Sample size =10, standard deviations are shown in the graph.

Figure 3. Ocular clusterin localization by immunohistochemistry and in situ analysis. Shown are sections of cornea (panels **A**-**C**, serial sections), skin/eyelid (panels **D**, **E**, serial sections), ciliary body (unfilled arrow and * in panel **F**), and lens (panels **G**, **H**, serial sections). Immunodetection with G7 are shown in panels **B**, **E**, **F**, and **H**; panels **A**, **D**, and **G** are the negative controls in which the G7 antibody was omitted. In situ hybridization of an anti-sense clusterin mRNA probe on cornea is shown in panel **C**. Panel **I**: graph illustrating the distribution of silver grain over different regions of the cornea shown in panel **C**, for each general region 10 independent counts within a fixed area were taken. Solid arrows indicate the interface between epithelial and stromal cells (cs) in the cornea, arrow heads indicate the area of epithelial cells in the lens.

showed a high level of clusterin mRNA. For the most part, our data obtained in monkey mirrored the profile of that seen in the few human tissues that had been examined by either immunohistochemistry or western analysis (Table 1). The only exception was our lack of detection of the protein in whole corneal lysates. The detection by immunohistochemistry of clusterin in sections of human cornea and western analysis of cell lysates made from isolated corneal epithelial cells had been previously reported [19].

In situ hybridization and immunohistochemistry: In order to confirm or refute published results, especially those for corneal tissue, we decided to undertake an analysis of human eye derived tissues. In our hands, in situ hybridization showed that clusterin mRNA is highly expressed in corneal epithelial cells and to a lesser extent in the stromal cells of the cornea (Figure 3C,I). The cornea sections used in the in situ hybridization and immunohistochemistry (Figure 3A-C) were serial sections. By a visual comparison, it becomes evident where on the slide there is no tissue, where the corneal epithelial layer is, and where the stromal regions are. We have done a relative comparison of the grain density over these three regions (Figure 3I). The average grain density over the cornea epithelial layer is four times greater the density over regions of the slide with no tissue (background), while the grain density over the stromal region is approximately 1.5 times that of background.

Immunohistochemical analysis showed a localization of clusterin protein in the epithelial cells and a lower level of clusterin in the stroma of the cornea (Figure 3B). This staining was not apparent in the control slides in which the primary antibody was omitted (Figure 3A). In other sections, we were also able to specifically localize clusterin protein to regions of the eyelid (Figure 3E), ciliary body (Figure 3F) and lens epithelial cells (Figure 3H).

Clusterin expression in RPE cells in culture: Levels of clusterin mRNA were greatly increased in cultured monkey RPE cells, compared to native RPE/choroid (Figure 1), and this elevation in expression was mirrored in the differential levels of clusterin detected by western analysis (Figure 2). This is notable because in other respects monkey RPE cells maintained under our in vitro conditions, as exemplified in Figure 4, demonstrate a wide array of attributes characteristic of RPE in vivo, such as de novo melanogenesis, expression of cellular retinaldehyde-binding protein, vectoral fluid transport (manifested as domes on impermeable supports) and morphological apical-basal polarity [22].

Using western analysis of apical and basal conditioned media that was generated over a period of two months by RPE cells cultivated on filter inserts, we found three primary bands detected by the clusterin-specific antibody, two HMW bands, and an *80 kDa species band (Figure 5). The *80 kDa species band runs with the same electrophoretic mobility as the single band in conditioned media from RPE cells maintained on tissue culture plastic (Figure 2). Results of densitometric and statistical analysis of the data reveal that the total clusterin is preferentially distributed in the basal compartment at a ratio of 1:2 apical to basal. Analysis of the *80 kDa band alone

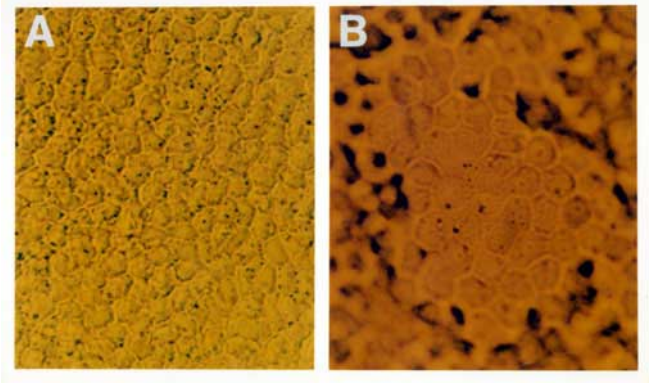


Figure 4. Morphology of polarized monkey RPE cells in culture. Panel **A** is a photomicrograph of first passage monkey RPE cells in a confluent monolayer culture using pseudo-interference optics. (Magnification: 350x). Panel **B** is a phase contrast photomicrograph of monkey retinal pigmented epithelial cells at confluence in first passage culture. RPE cells comprising the apex of a fluid-filled dome are in focus, with surrounding cells attached to the plastic culture dish beyond the plane of focus. The appearance of domes in epithelial cultures is emblematic of vectorial ion and small molecule transport, generating hydrostatic pressure from fluid movement to a basal compartment between the monolayer of cells and the impermeable plastic substrate, counterbalanced by the resistance of circumferential tight junctions linking the cells. (Magnification: 350x).

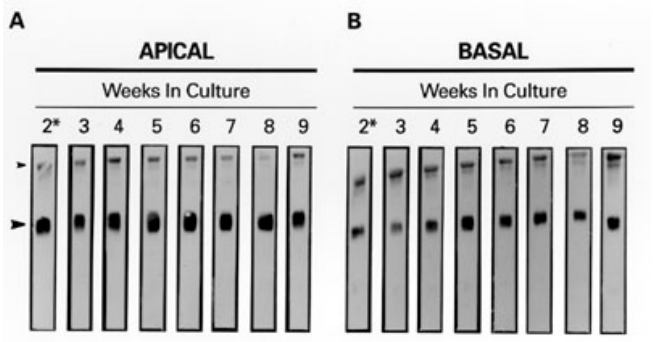


Figure 5. Analysis of apical and basal clusterin from polarized monkey RPE cells in culture. RPE cells were cultivated on filter inserts as described in Methods. Shown are photographs of the immunostained blots of conditioned media samples taken at weekly intervals starting after the initial two weeks in culture. Equivalent volumes of apical (**A**) and basal (**B**) medium per sampling were resolved by 12.5% SDS-PAGE and subsequent western analysis using the G7 anti-clusterin antibody at a dilution of 1:1. Two subsets of bands are detected, a set that represents high molecular weight clusterin species (HMW, >100 kDa), and an 80 kDa clusterin species (*80 kDa). Densitometric and statistical analysis of the clusterin immunoreactivity indicate that the clusterin secretion profiles do not change over time. Total clusterin secretion favors a slightly basal profile (1:2 apical:basal), the *80 kDa species considered alone is more equally bi-directionally secreted (1:1.5 apical:basal), and the HMW species is predominantly secreted (1:3.3 apical:basal). Small arrow head: HMW clusterin species. Large arrow head: *80 kDa clusterin species.

shows that this clusterin species is more evenly distributed between the two media compartments, with an apical:basal ratio of 1:1.5. Analysis of the two HMW bands together suggests that there is a higher bias towards basal secretion of these variants (1:3.3, apical:basal, respectively). Statistical analysis for goodness of fit (χ^2 test, α=0.05, df=20) indicates that the ratios of apical:basal clusterin did not vary significantly over the sampling period. The apical RPE conditioned media consisted of 67% *80 kDa clusterin and 33% HMW clusterin (a 2:1 ratio). The basal RPE conditioned media consisted of 51% *80 kDa clusterin and 49% HMW clusterin (a 1:1 ratio).

DISCUSSION

This is the first study that takes a concentrated look at clusterin expression in monkey-derived eye tissues, and it is the first study to examine eyelid, iris, limbus, and orbital muscle from any species for clusterin expression. In addition, some of our data clarify and extend our understanding of clusterin in a number of different eye tissues. For example, it had been shown previously by immunohistochemistry that high levels of clusterin protein are present in the ciliary body. Localization of clusterin mRNA to the tissue by in situ hybridization, however, was obscured by the high level of pigment present in the tissue [17]. The results of our northern blot analyses clearly demonstrate that clusterin mRNA is present at high levels in the ciliary body and our western analyses show that ciliary body tissue lysates have both types of clusterin, HMW and *80 kDa species. On a broader scale, our study demonstrates that the wide range of clusterin expression in different tissues observed in human eyes is also conserved in the monkey eye.

Unique to our study is the observation of two distinct subsets of clusterin protein in monkey eye tissues, a set of very high molecular weight species and a set of proteins of approximately 80 kDa in size. The size of clusterin mRNA is identical in all eye tissues that express it. This suggests that the differences in the molecular sizes of clusterin protein are due to tissue specific differences in post-translational processing of the protein. Clusterin has a number of conserved putative glycosylation sites and is prone to other forms of modification, such as sulphation [1-4]. Based on the *80 kDa region protein bands alone there are at least 5 different eye-derived clusterin species that can be differentiated by slight differences in electrophoretic mobility in this size region. The HMW bands that are detected are likely to represent additional clusterin forms. In plasma, clusterin can aggregate with high density lipoproteins forming complexes in excess of 200 kD [34]. In chromaffin granules, clusterin is present in membrane associated and membrane unassociated isoforms and it is the membrane associated form that is prone to aggregate into higher molecular weight multimers [35]. To our knowledge, our observations of different clusterin forms in different ocular tissues represents the first time that this has been clearly demonstrated. The appearance of altered forms of clusterin is intriguing because alterations in protein structure may lead to alterations in specific function, suggesting that tissue specific differences in clusterin structure may underscore tissue specific functions. The exact nature of the post-translational modification(s) that gives rise to the different isoforms of the protein observed is not known, and will require additional biochemical analysis to determine.

For the most part, the tissues that we observe clusterin mRNA and protein to be present in coincide with published reports of clusterin expression in the corresponding tissue in humans [17]. The only exception to this is our results for the cornea, which differ slightly from those reported by Nishida et al. [19]. In that study, clusterin mRNA was found to be highly abundant in the corneal epithelium by in situ hybridization; clusterin protein was found to be present by western analysis of isolated corneal epithelium cells and was localized to the apical epithelial cells by immunohistochemistry. In our study we verified, by northern analysis, that clusterin mRNA is abundant in corneal tissue. Results of in situ hybridization, using a radioactive probe (which is more sensitive than the biotinylated clusterin probe used in the Nishida et al. study), showed that clusterin mRNA is present in the corneal epithelium and is present at lower levels in the stroma. Our immunolocalization of clusterin protein mirrors this and shows localization of the protein to the entire corneal epithelium with much lower levels in the stroma. In further contrast to Nishida et al. who detected clusterin in western analysis of isolated corneal epithelial cells, we did not detect clusterin by western analysis of whole cornea lysates. Our explanation for this discrepancy is that the cornea is a common and clinically important site for lipid deposition [36] and clusterin can associate with high density lipoproteins (HDL) and become insoluble. The biological function of this association is that clusterin can function to facilitate transport of lipids among cells within an organ or tissue [9,37]. It is possible that in whole cornea lysates, clusterin becomes lipid associated and is lost in our lysate preparation for western analysis, a scenario that would not happen for isolated corneal epithelial cells.

The detection of clusterin in unconcentrated vitreous samples indicates that clusterin is a major component in these fluids. The clusterin that is present in the vitreous is eye derived [17], but the actual tissue source of this vitreal clusterin is not known. A number of RPE and retinal secreted proteins, including PEDF, IRBP, and TGF-βhave been found in the vitreous [26,36; B. Wiggert, personal communication]. Clusterin is relatively abundant in RPE, and it is thus possible that the RPE may also secrete clusterin into the vitreous. Analysis of conditioned media from retinal pigment epithelial (RPE) cells in culture indicate that additional modifications to the protein occur prior to secretion. The clusterin *80 kDa band detected in the conditioned media is larger than the band detected in the RPE/choroid tissue lysate.

The function of epithelial cells is two-fold. First, they generate and maintain boundaries between distinct regions within an organ. Second, they condition the micro-environment on both sides of the epithelial layer by their capacity to differentially secrete and transport selected substrates. In the case of RPE cells cultured on a permeable support, the stable secretion profile of the prominent *80 kDa band mirrors an almost equal balance between apical and basal release, while the HMW pair of variants show a preferentially basal secretion

profile. In contrast, we have observed an almost exclusive apical secretion of another protein, PEDF, in this same system (unpublished observation). The pattern of secretion observed for clusterin is therefore specific for this protein. A predominant basal secretion of clusterin in models of vectorial secretion has not been previously demonstrated. A well studied model for examining the process of vectorial secretion is polarized Madin-Darby canine kidney (MDCK) epithelial cells in culture. Clusterin secretion in the MDCK system occurs constitutively from the apical surface [11]. Inhibition of N-glycosylation by addition of tunicamycin does alter the secretion profile of clusterin from these cells, transforming the normal constitutive apical secretion profile to an equal apical:basal secretion pattern [11]. These observations would suggest that the regulation or extent of N-glycosylation in polarized MDCK cells is different from polarized RPE cells. The RPE has already been shown to be exceptional with respect to its apical membrane expression of Na, K-ATPase and associated cytoskeletal components [38-40]. In vivo, basal secretion of the HMW forms of clusterin, which might function to aggregate with lipids or lipoproteins towards the choroid and perhaps into the blood stream, may function as a carrier/redistribution mechanism for lipid turnover. Finally, the two HMW species of clusterin that are present in the conditioned medium from RPE cells cultured on permeable filters are absent in RPE monolayers cultured on nonpermeable tissue culture plastic substrates, suggesting that the former respond appropriately to the apical and basal micro-environments that they maintain.

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