Clusterin Deficiency in Eyes with Pseudoexfoliation Syndrome May Be Implicated in the Aggregation and Deposition of Pseudoexfoliative Material

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PURPOSE. To determine the role of the extracellular chaperone clusterin in the pathophysiology of pseudoexfoliation (PEX) syndrome/glaucoma, which is characterized by the stable deposition of abnormal extracellular fibrillar material in anterior segment tissues.

METHODS. Real-time PCR, in situ hybridization, and immunohistochemistry were applied to analyze the mRNA and protein expression of clusterin in PEX eyes of patients without and with glaucoma and to compare them with eyes of patients with primary open-angle glaucoma and angle-closure glaucoma and with normal control eyes. Aqueous levels of clusterin were determined by Western blot analysis. Real-time PCR and Western blot analysis were used to study the effect of TGF-β1, which is significantly increased in the aqueous humor of PEX eyes, on clusterin expression by nonpigmented ciliary epithelial cells in vitro.

RESULTS. Clusterin mRNA was ubiquitously expressed in most ocular cells and tissues, particularly in the epithelium of ciliary processes, whereas the protein was mostly located to extracellular structures, such as ocular basement membranes and stromal fibers. Real-time PCR and in situ hybridization displayed significant downregulation of clusterin mRNA in all anterior segment tissues of PEX eyes, irrespective of the presence or type of glaucoma, compared with normal and glaucomatous control eyes, whereas posterior segment tissues did not show any differential expression. A generally decreased immunoreactivity, but a prominent binding of clusterin to all PEX deposits, could be observed in ocular tissues of PEX eyes. Clusterin levels in aqueous humor were significantly reduced in eyes of patients with PEX syndrome compared with normal and glaucomatous control eyes. The expression of clusterin mRNA and protein in nonpigmented ciliary epithelial cells was significantly downregulated by TGF-β1 in vitro.

CONCLUSIONS. Considering the known role of clusterin as a highly efficient extracellular chaperone, its deficiency in the anterior segment of PEX eyes may promote the stress-induced aggregation and stable deposition of the pathologic extracellular matrix product characteristic of PEX syndrome. (Invest Ophthalmol Vis Sci. 2006;47:1982–1990) DOI:10.1167/iovs.05-1580

Pseudoexfoliation (PEX) syndrome, which represents the most commonly identified specific cause of open-angle glaucoma, is a generalized disorder of the extracellular matrix and is characterized by the intraocular and extraocular production and accumulation of abnormal fibrillar extracellular material (PEX material). Progressive obstruction of the trabecular meshwork by locally produced PEX material is considered the primary cause of chronic pressure elevation and glaucoma development in eyes of patients with PEX syndrome. Although the exact biochemical composition of the pathologic matrix product is still unknown, previous (immuno)histochemical studies have shown PEX material to represent a highly glycosylated, cross-linked, and enzymatically resistant glycoprotein–proteoglycan complex bearing epitopes of the basement membrane and the elastic fiber systems. Although PEX syndrome is a systemic condition, the characteristic fibrillar deposits are primarily deposited on ocular structures that line the aqueous-bathed surfaces of the anterior segment. The composition of the aqueous humor may therefore, crucially influence the matrix metabolism in anterior segment tissues of PEX eyes. A hallmark of PEX syndrome is the significantly elevated level of fibrogenic transforming growth factor-β1 (TGF-β1) in aqueous humor and its upregulation in anterior segment tissues of PEX patients. In addition, an imbalance of matrix metalloproteinases (MMPs) and their inhibitors (tissue inhibitors of metalloproteinases [TIMPs]), a decrease in antioxidative protective factors (ascorbic acid) and a concomitant increase in oxidative stress markers (8-isoprostaglandin-F2α), and a significantly reduced oxygen partial pressure have been documented in aqueous humor samples of PEX patients.

Recently, we provided evidence that the mRNA expression of clusterin, which is present at high concentrations in normal aqueous humor, is downregulated in the iris, lens, and ciliary processes of patients with PEX syndrome. Clusterin or apolipoprotein J, named for its ability to induce aggregation (clustering) of blood cells in vitro, is a multifunctional 70- to 80-kDa glycoprotein constitutively produced and secreted by almost all cell types and is found in all body fluids. In the eye, clusterin has been shown to be present in the cornea, lens, ciliary body, retina, and aqueous and vitreous humor, and has been implicated in retinal (patho)physiology based on its upregulation in retinitis pigmentosa, after retinal reperfusion injury, and after light-induced photoreceptor cell degeneration. A number of physiologic functions have been proposed for clusterin, including implications in apoptosis and complement regulation, protection of cell membranes, stabilization of cell–cell and cell–matrix interactions, and inhibition of stress-induced precipitation and aggregation of misfolded proteins through its action as an extracellular chaperone. Clusterin expression is, therefore, strongly induced under cellular stress conditions, such as heat shock and oxidative stress in vitro, and in an impressive array of pathologic conditions.
including glomerulonephritis, Alzheimer disease, and myocardial infarction.\textsuperscript{16} Clusterin has been further associated with a wide variety of pathologic extracellular deposits, among them drusen,\textsuperscript{17} arteriosclerotic plaques,\textsuperscript{18} and senile plaques in Alzheimer disease,\textsuperscript{19} suggesting a role for this chaperone in the formation of abnormal extracellular matrix deposits typical of PEX syndrome.

Therefore, the purpose of this study was to investigate the expression of clusterin in eyes of patients with PEX syndrome without and with glaucoma in comparison with normal and glaucomatous control eyes. In particular, we analyzed the expression of clusterin on the mRNA and protein levels in ocular tissues and assessed the protein content of clusterin in aqueous humor samples. In addition, we studied the effect of TGF-\(\beta\) on clusterin expression by ciliary epithelial cells in vitro. We provide evidence for a selective downregulation of clusterin expression in anterior segment tissues and significantly reduced aqueous levels of clusterin in eyes of patients with PEX syndrome. Furthermore, the in vitro downregulation of clusterin mRNA and protein by TGF-\(\beta\) provides a possible explanation for the reduced expression of clusterin in anterior segment tissues of PEX eyes. The findings suggest that the accumulation of the characteristic pathologic matrix product in PEX eyes may partly arise from stress-induced protein misfolding and aggregation promoted by a distinct deficiency of an important extracellular chaperone.

**METHODS**

**Tissues and Samples**

For RNA isolation, ocular tissue were obtained from three eyes of patients with PEX-associated open-angle glaucoma (age, 82.7 ± 5.4 years; two women, one man), three eyes of patients with PEX-associated angle-closure glaucoma (age, 86.3 ± 7.4 years; two women, one man), three eyes of patients with primary open-angle glaucoma (POAG; age, 76.7 ± 7.8 years; two women, one man), and three eyes of patients with secondary angle-closure glaucoma without PEX (age, 84.7 ± 4.1 years; two women, one man). Eyes had to be surgically enucleated because of painful absolute glaucoma. Immediately after enucleation, tissue was prepared under a dissecting microscope and was shock frozen in liquid nitrogen in RNA extraction buffer. In addition, we used three eyes of donors with PEX syndrome without glaucoma (age, 77.0 ± 6.2 years; one woman, two men), three normal-appearing eyes (age, 75.3 ± 10.1 years; one woman, two men) of donors without PEX syndrome or any other known ocular disease, and three pairs of eyes (age, 81.0 ± 1.6 years; one woman, two men) of donors with clinically and macroscopically unilateral PEX syndrome that were obtained at autopsy and processed within 8 hours of death.

For in situ hybridization, another four eyes of patients with PEX-associated open-angle glaucoma (age, 77.5 ± 4.9 years; three men, one woman), one eye of a man with PEX-associated angle-closure glaucoma (age, 83 years), four eyes of patients with absolute POAG (age, 78.0 ± 6.7 years; two men, two women), and one eye of a man with secondary angle-closure glaucoma (age, 80 years) without evidence of PEX syndrome were obtained by surgical enucleation. The eyes were fixed in buffered 4% paraformaldehyde immediately after enucleation for optimal preservation of RNA. In addition, we included one eye of a male donor with PEX syndrome without glaucoma (age, 64 years) and one normal-appearing eye (age, 64 years) of a male donor without PEX syndrome or any other known ocular disease that were obtained at autopsy and fixed within 8 hours of death.

Immunohistochemistry was performed on five eyes of donors with PEX syndrome (age, 72.1 ± 6.9 years; two men, three women) and five normal-appearing eyes of donors without PEX (age, 70.5 ± 6.5 years; two men, three women) that were obtained at autopsy and fixed within 10 hours of death.

**Clusterin Deficiency in Pseudoexfoliation Syndrome**

Aqueous humor was aspirated intraoperatively from 12 patients with PEX syndrome without glaucoma (age, 76.1 ± 7.4 years), 12 patients with PEX glaucoma (age, 76.4 ± 7.3 years), 12 patients with POAG (age, 71.3 ± 7.3 years), and 20 cataract patients without PEX syndrome or glaucoma (age, 74.1 ± 5.6 years) during cataract or filtration surgery. One hundred microliters aqueous humor was withdrawn through an ab externo limbal paracentesis site with a 27-gauge needle on a tuberculin syringe. The samples were immediately frozen in liquid nitrogen and stored at −80°C. Patients who had ophthalmic diseases other than glaucoma or cataract or who had undergone previous surgery were excluded from the study. Serum samples were also collected from 12 patients in each group and were immediately frozen in liquid nitrogen and stored at −80°C. Informed consent to tissue and aqueous humor donation was obtained from the patients or, in case of autopsy eyes, from their relatives. The study protocol was approved by the local ethics committee and adhered to the tenets of the Declaration of Helsinki for experiments involving human tissue and samples.

**Real-Time RT-PCR**

Total RNA was isolated from cornea, trabecular meshwork, iris, lens epithelium, ciliary processes, retina, and choroid specimens with the use of a commercially available kit (RNeasy kit; Qiagen, Hilden, Germany), which included an on-column DNase I digestion step, in accordance with the manufacturer’s instructions. First-strand cDNA synthesis was performed using 1 μg total RNA, 200 U Superscript II reverse transcriptase (Invitrogen, Karlsruhe, Germany), and 500 ng oligo dT primers (Roche Diagnostics, Mannheim, Germany) in a 20-μL reaction volume. Gene-specific primers (MWG Biotech, Anzing, Germany) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; upstream, AGC TCA CTG CTA TGG CCT TC; downstream, AGC CCT GCT TCA CCA CCT TC; GenBank accession number M33197; http://www.ncbi.nlm.nih.gov/GenBank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD) and human clusterin (upstream, TCC AGG AAA TGT CCA ATC AGG; downstream, GAG CAG TGT CTT GCC CTC TTC; accession number NM_001831) were designed to anneal with sequences located in different exons by means of software (Primer 3).\textsuperscript{20} Identity of PCR fragments was subsequently confirmed by sequence analysis (Prism 3.0 sequence analyzer; Applied Biosystems, Foster City, CA).

Quantitative real-time PCR was performed (iCycler IQ Thermal Cycler; Bio-Rad, Munich, Germany). A typical PCR reaction (25 μL) contained 2 μL of the 1:500 diluted first-strand product (corresponding to 0.2 ng total RNA), 0.4 μM each of upstream and downstream primer, 3.5 mM MgCl\(_2\) (GAPDH) or 4 mM MgCl\(_2\) (clusterin), respectively, and iQ SYBR Green Supermix (Bio-Rad) according to the manufacturer’s instructions. All samples were analyzed in triplicate using a program with an initial denaturation step of 95°C for 5 minutes and 40 cycles of 95°C for 30 seconds, 64°C for 30 seconds, and 72°C for 30 seconds. For quantification, standard curves using serial dilutions (10\(^{-1}\)–10\(^{-7}\) copies) of plasmid-cloned specific amplification products were run in parallel, and amplification specificity was checked using melting curve analysis. For standardization of levels of gene expression, mRNA ratios relative to the housekeeping gene GAPDH were calculated.

**In Situ Hybridization**

In vitro transcription of the linearized construct, a 445-bp cDNA fragment (nts, 178–619; accession number, NM_001831) cloned into pCRII TOPO (Invitrogen, Carlsbad, CA), was performed with SP6 and T7 RNA polymerase in the presence of digoxigenin-11-uridine triphosphate (DIG-UTP) to produce DIG-labeled, single-strand antisense or sense RNA probes (DIG RNA Labeling Kit; Roche Diagnostics) according to the manufacturer’s instructions. The quality of the transcripts was controlled by using denaturing formaldehyde–agarose gels before and after DNase I digestion. Hybridization of 4-μm paraffin sections of six eyes of patients with PEX syndrome and six control eyes was
performed as previously described. Hybridization with sense strand riboprobes served as negative controls, whereas hybridization with 18S rRNA antisense probes served as positive controls.

**Western Blot Analysis**

Aqueous humor samples, serum samples, and cell culture supernatants were collected, and total protein concentrations were determined by Bradford protein assay (Bio-Rad) with bovine serum albumin as a standard. Cell culture supernatants were 10-fold and aqueous samples were 5-fold concentrated by centrifugation using centrifugal filters (Amicon; 10kDa cutoff; Millipore, Bedford, MA), in accordance with the manufacturer’s instructions. Serum samples were diluted 1:100 in PBS. A 10-μL volume of each sample was separated by SDS-PAGE and transferred onto nitrocellulose membranes (Hybond ECL; Amersham Biosciences, Freiburg, Germany) with a semidyblotting unit (Bio-Rad) at 25 V for 2 hours in 1× transfer buffer (50 mM Tris, 20% methanol, 0.3% glycerin, 0.04% SDS). Membranes were blocked with 5% bovine serum albumin in PBS/0.1% Tween-20 (PBST) for 1 hour and incubated overnight at 4°C with mouse monoclonal antibodies against human clusterin (clone E5; BD Biosciences, Heidelberg, Germany; or clone Hs-3; Biovendor, Heidelberg, Germany) diluted 1:100 or 1:400 in PBST, respectively. Horseradish peroxidase–conjugated goat anti-mouse IgG (Bio-Rad) at a dilution of 1:6000 in PBST was used as secondary antibody. In negative control experiments, the primary antibody was replaced by PBS or equimolar concentrations of an irrelevant primary antibody. For detection of bands, a Western blot detection system (Fluor-S-Max; Bio-Rad).

**Immunohistochemistry**

Light microscopic indirect immunofluorescence labeling was performed on ocular tissue sections of PEX and control eyes, as previously described. Two mouse monoclonal antibodies against human clusterin were used. Antibody binding was detected by Cy3-conjugated secondary antibodies (Molecular Probes, Eugene, OR). In negative control experiments, the primary antibody was replaced by PBS or equimolar concentrations of an irrelevant primary antibody.

**Cell Culture**

To study the effect of TGF-β1 on the expression of clusterin, the immortalized human nonpigmented ciliary epithelial cell line OD-M25 was used at passage 17. Cells were maintained in DMEM (Gibco BRL, Grand Island, NY) containing 10% fetal calf serum (FCS) and 50 μg/mL gentamicin in a 95% air/5% CO2 humidified atmosphere at 37°C. Cells were grown to subconfluence and then were treated with 1 ng/mL TGF-β1 (R&D Systems, Wiesbaden, Germany) for 6, 12, 24, 48, and 72 hours. Cultures without added TGF-β1 served as controls. Cells were harvested, total RNA was extracted, and cell culture supernatants were analyzed by Western blot analysis, as described earlier.

**Statistical Analysis**

Data are presented as mean ± SD. Statistical evaluation of significant differences between groups of patients was performed with the Student’s t test for pairwise comparison. Pearson correlation analysis was used to determine a potential correlation between the total protein concentrations and the levels of clusterin in aqueous humor samples. P < 0.05 was considered statistically significant.

**RESULTS**

**Clusterin mRNA Expression in Ocular Tissues**

Quantitative real-time PCR showed a high basal mRNA expression of clusterin in human ocular tissues from normal and glaucomatous control eyes (Fig. 1A). A particularly pronounced expression level, which was approximately 80-fold higher than that of the housekeeping gene GAPDH, was found in ciliary processes of all control eyes without PEX. In all PEX eyes examined (n = 4), clusterin expression was markedly downregulated in anterior segment tissues, most prominently in ciliary processes specimens, compared with control eyes without PEX (n = 4; Fig. 1A). Differences in expression levels were statistically significant in the cornea (3.7-fold; P < 0.05), the trabecular meshwork (2.9-fold; P < 0.05), the iris (5.1-fold; P < 0.0001), the lens (1.8-fold; P < 0.002), and the ciliary processes (6.8-fold; P < 0.0001). In contrast, posterior segment tissues, such as retina and choroid, did not show any differential expression between PEX and control eyes.

The downregulation of clusterin mRNA appeared to be independent of the presence or type of glaucoma because it was consistently found in ciliary process tissue of all groups of PEX-positive eyes but was unchanged in retina and choroid. Values are mean ± SD of four separate experiments comparing PEX samples (PEX syndrome, unilateral PEX syndrome, PEX-associated open-angle glaucoma [POAG], and PEX-associated angle-closure glaucoma [ACG]) and control samples (normal donor eye, unaffected fellow eye in unilateral PEX syndrome, POAG, and ACG) (n = 4 for each tissue; t < 0.05; **P < 0.001; ***P < 0.002). (B) Reduced clusterin mRNA expression in ciliary processes of PEX-positive eyes irrespective of the presence or type of glaucoma. In unilateral PEX syndrome, clusterin mRNA expression is reduced in the PEX-positive eye and displays normal levels in the unaffected fellow eye. Values are mean ± SD of three separate experiments comparing PEX samples (PEX syndrome, POAG, and ACG) (n = 3 for each group; *P < 0.02; **P < 0.001; ***P < 0.005).
PEX patients, such as in eyes of patients with PEX syndrome without glaucoma (n = 3) compared with age-matched normal eyes (n = 3; P = 0.02), in eyes of patients with PEX-associated open-angle glaucoma (n = 3) compared with eyes of patients with POAG (n = 3; P < 0.005), and in eyes of patients with PEX-associated angle-closure glaucoma (n = 3) compared with eyes of patients with angle-closure glaucoma without PEX (n = 3; P < 0.001; Fig. 1B). In another three pairs of eyes of patients with clinically unilateral PEX syndrome, clusterin mRNA expression was reduced in the affected eyes, whereas it displayed normal levels in the unaffected fellow eyes (P < 0.001; Fig. 1B).

To further determine the localization of clusterin mRNA expression within the tissues, we performed in situ hybridization on sections of PEX (n = 6) and control (n = 6) eyes. Signals for clusterin mRNA were localized to all ocular tissues of PEX and control eyes. In particular, clusterin mRNA was found to be expressed in cells of the corneal and conjunctival epithelia, particularly in their basal cell layers; in the ciliary and pre-equatorial lens epithelia; in vascular endothelial cells of the conjunctiva, iris, and ciliary body; in corneal and trabecular endothelial cells; in some cells of the corneal, conjunctival, iridal, and ciliary stroma; in iridal and ciliary muscle cells; in all layers of the retina; and in choroidal cells. Signals were, however, distinctly weaker in anterior segment tissues of PEX eyes, particularly in the nonpigmented epithelium of the ciliary processes and in iridal stromal cells (Fig. 2A–D). Hybridization with the sense probe for clusterin served as negative control and yielded no signals, whereas hybridization with the 18S rRNA antisense probe served as positive control and resulted in strong and ubiquitously distributed signals throughout all ocular tissues (data not shown).

Clusterin Protein Expression in Ocular Tissues

By light microscopic immunohistochemistry, clusterin protein could be immunolocalized to extracellular structures—such as basement membranes of the corneal and conjunctival epithelia, the iris pigment epithelium, the pigmented and nonpigmented ciliary epithelia, and the periphery of the trabecular beams—in eyes with and without PEX syndrome. The protein could be further localized to blood vessel walls in the stroma of the conjunctiva, iris, and ciliary body and to the wall of Schlemm’s canal. Moreover, extracellular fiberlike structures, probably representing elastic fibers, in the conjunctival, iridal, and ciliary stroma and within iridal and ciliary muscles were positively labeled. There was, however, no evidence of cellular staining or staining of zonular fibers. In all PEX eyes examined, immunolabeling for clusterin in extracellular structures was slightly reduced, particularly in iris and ciliary body tissues (Fig. 3A–D). However, a prominent binding of clusterin to all PEX material deposits on ocular surfaces and within ocular tissues was evident (Figs. 3B, 3D, 3E). Antibody binding was abolished when an irrelevant monoclonal antibody or PBS was used instead of the primary antibodies (Fig. 3F).

Clusterin in Aqueous Humor

To determine the relative abundance of clusterin in aqueous humor and serum samples from patients with cataract, POAG, PEX syndrome, and PEX-associated open-angle glaucoma (12 patients each), Western blot analyses using two different antibodies were performed. Clusterin was detected in considerable amounts in serum samples from all groups of patients as a band between 70 and 80 kDa, displaying only minimal interindividual variability and virtually no difference in band intensities between groups of patients (data not shown). In aqueous humor samples, Western blot analysis also identified a prominent immunoreactive band between 70 and 80 kDa (Fig. 4A).

Both antibodies yielded similar findings. Clusterin immunoreactivity was abolished when an unrelated monoclonal antibody or PBS was used instead of the primary antibody (data not shown). In spite of high interindividual variability, the amount of clusterin was significantly reduced in aqueous humor samples from patients with PEX syndrome compared with cataract (3-fold; P < 0.001) and POAG (3-fold; P < 0.0001; Fig. 4B). In contrast, samples from patients with PEX glaucoma displayed a small but significant increase in clusterin levels compared with cataract (1.6-fold; P < 0.02) and POAG (1.6-fold; P < 0.06; Fig. 4B).

Total protein concentration was examined to assess blood-aqueous barrier function and measured 197 ± 85 µg/mL in cataract, 245 ± 118 µg/mL in PEX syndrome, 214 ± 96 µg/mL in POAG, and 565 ± 228 µg/mL in PEX glaucoma patients. The difference between patients with PEX glaucoma and all other groups of patients was statistically significant (P < 0.001). A significant correlation between the total aqueous protein concentration and the amount of clusterin (r = 0.873; P < 0.001) could only be established for aqueous humor samples from patients with PEX glaucoma.

Effects of TGF-β1 on Clusterin mRNA and Protein Expression In Vitro

In the search for pathogenetic factors that could be responsible for clusterin downregulation in anterior segment tissues of PEX eyes, we studied the effect of TGF-β1 on clusterin mRNA expression and secretion in vitro. Human nonpigmented ciliary epithelial cells (ODM-2) were treated with 1 ng/mL TGF-β1 for up to 72 hours under serum-free conditions. Cells maintained in serum-free medium without growth factor served as controls.

Quantitative real-time PCR demonstrated that clusterin mRNA expression was significantly downregulated (1.7-fold; P < 0.015) after 12 hours of exposure to TGF-β1 (Fig. 5A). Maximum level of downregulation was reached after 48 hours (8-fold; P < 0.001). Consistently, Western blot analysis of cell culture supernatants showed a significantly decreased level of clusterin protein (1.5-fold; P < 0.05) after 24 hours of TGF-β1 exposure with a maximum level of downregulation after 72 hours of exposure (4.8-fold; P < 0.006; Fig. 5B).

Discussion

Structural and Functional Properties of Clusterin

Clusterin is a secreted disulfide-linked heterodimeric glycoprotein of 70 to 80 kDa; 50% of its mass is N-linked carbohydrate of variable structure. It is synthesized as a single primary polypeptide chain of 449 amino acids and is internally cleaved into its α- and β-chains, which remain linked by five disulfide bridges, before secretion from the cell.23 Sequence analyses predict four heparin-binding domains that may mediate interactions with cell membranes and the extracellular matrix and four amphipathic helical regions that may allow binding to hydrophobic molecules.16 Clusterin is highly conserved among species (70%–80% in mammals), found in all biologic fluids, and expressed in most tissues, primarily in epithelial cells at fluid–tissue boundaries.24 Clusterin has been implicated in a variety of biologic processes, including lipid transportation, cell membrane remodeling, endocrine secretion, complement inhibition, and regulation of apoptosis.25 Its high degree of sequence conservation, its almost ubiquitous tissue distribution, and the absence of functional clusterin polymorphisms in humans suggest that the protein accomplishes a function of fundamental biologic importance.

In response to cellular stress conditions including heat shock, UV radiation, and oxidative stress, and in a wide array of
FIGURE 2. Expression of clusterin mRNA in tissues from control (A, C) and PEX (B, D) eyes, both with open-angle glaucoma, as detected by in situ hybridization. Positive signals (arrows) are indicated by purple-blue staining, in contrast to the brownish lipofuscin and black melanin pigment of the cells. (A, B) Decreased expression of clusterin in the nonpigmented ciliary epithelium of PEX eyes. (C, D) Decreased expression of clusterin in iridal stromal cells of PEX eyes. BV, blood vessel; CE, ciliary epithelium; CS, ciliary stroma; IPE, iris pigment epithelium; IS, iris stroma; PEX, PEX material. Original magnification, ×200.

FIGURE 3. Immunofluorescence labeling of clusterin protein in ocular tissues of normal control eyes (A, C) and eyes of patients with PEX syndrome (B, D, E). Positive signals are indicated by red fluorescence in contrast to blue fluorescence of DAPI-stained nuclei. (A, B) Compared with control eyes, eyes of patients with PEX syndrome show slightly reduced immunostaining of both basement membranes (arrows) of the ciliary epithelium but prominent labeling of PEX material deposits on the surfaces of ciliary processes. (C, D) In iris tissue, PEX eyes reveal a slightly reduced immunoreactivity of the dilator muscle but prominent labeling of iridal blood vessel walls and PEX material deposits on the surface of the iris pigment epithelium. (E) In conjunctival specimens of PEX eyes, immunopositive PEX material deposits are observed in the periphery of stromal vessels in addition to positive stromal elastic fibers. (F) Negative control section of ciliary body tissue using PBS instead of the primary antibody. BV, blood vessel; CE, ciliary epithelium; CS, ciliary stroma; DIL, dilator muscle; EF, elastic fibers; EP, conjunctival epithelium; IPE, iris pigment epithelium; IS, iris stroma; PC, posterior chamber; PEX, PEX material. Original magnification, ×200.
pathologic conditions, such as glomerulonephritis, neurodegenerative disorders, cancer, multiple sclerosis, atherosclerosis, and myocardial infarction, clusterin expression is markedly upregulated in vitro and in vivo.\textsuperscript{15–19,25–27} Evidence suggests that clusterin exerts a cytoprotective function in these stress conditions, which may be mediated by protection against oxidative stress, inhibition of apoptosis and complement-mediated cell lysis, promotion of cellular contacts, and protection of cell membranes.\textsuperscript{19} Inhibition of clusterin by antisense strategies significantly increased the cellular sensitivity to heat shock or oxidative stress in vitro.\textsuperscript{15,26} Transgenic mice overexpressing clusterin showed significantly less damage after ischemic brain injury, whereas clusterin knock-out mice displayed significantly greater damage.\textsuperscript{29} Finally, exogenously added clusterin can protect cells from cell death mediated by oxidative and heat stress, suggesting that clusterin exerts at least part of its protective effect upon secretion.\textsuperscript{30}

More recently, it has been proposed that clusterin represents a new class of highly efficient chaperones, similar to the small heat shock proteins; for clusterin, however, the main site of action is in the extracellular space.\textsuperscript{14,31–33} Molecular chaperones serve as a cellular rescue system by interacting with partially folded or unfolded stressed proteins to prevent their aggregation and precipitation and to encourage correct protein refolding. Misfolding of proteins is a result of structural modification typically caused by oxidative cell injury or mutation. Clusterin binds to exposed hydrophobic regions of a broad range of partially unfolded proteins producing solubilized high molecular-weight complexes, thereby preventing their aggregation and precipitation.\textsuperscript{31–33} Further, clusterin has been shown to preferentially interact with slowly aggregating target proteins.\textsuperscript{34} Although clusterin does not effect the refolding of destabilized proteins, it stabilizes them in a state competent for refolding into functional proteins by other chaperones, such as heat shock protein 70.\textsuperscript{70,9,52,54}

Expression of Clusterin in the Human Eye

In the adult human eye, clusterin has been shown to be present in the cornea, particularly in the corneal epithelium and endothelium, the conjunctival epithelium, the lens epithelium, the ciliary body, the retina, particularly in the inner nuclear and ganglion cell layers, and in aqueous and vitreous humor.\textsuperscript{7,10,13,35–37} The average concentration of clusterin in the aqueous humor showed high interindividual variations and measured $752 \pm 519 \text{ ng/mL}$, which is lower than in other biologic fluids, such as cerebrospinal fluid or seminal fluid.\textsuperscript{7} There was no correlation between the aqueous level of clusterin with the total aqueous protein concentration, supporting the assumption that most clusterin in the aqueous is synthesized in the eye, mainly by the ciliary body.\textsuperscript{36}

![Figure 4](https://iovs.arvojournals.org/)

**Figure 4.** Determination of clusterin protein levels in human aqueous humor by means of Western blot analysis. (A) Representative Western blot of aqueous humor samples from patients with cataract (1, 2), PEX syndrome (3, 4), POAG (5, 6), and PEX-associated OAG (7, 8) using clusterin monoclonal antibody and ECL detection. For normalization, each blot included one lane (9) with normal aqueous humor (NAH, mixture of 20 individual samples from cataract patients). (B) Intensities of specific immunoreactive bands were quantified by computerized densitometry. Signal intensity of NAH was set to 100%, and results are expressed as percentage of NAH, together with the relative change in aqueous clusterin levels compared with cataract. Densitometric values are mean ± SD for each patient group ($n = 12$). Aqueous levels of clusterin were significantly decreased in PEX syndrome (3-fold; $^*P < 0.001$) and displayed an increase in PEX-associated OAG (1.6-fold; $^{**}P < 0.02$) compared with cataract.

![Figure 5](https://iovs.arvojournals.org/)

**Figure 5.** Downregulation of clusterin mRNA (A) and protein expression (B) in cultured human nonpigmented ciliary epithelial cells (ODM-2) by TGF-β1. Values are mean ± SD of three separate experiments ($n = 5$). (A) Effect of 1 ng/mL TGF-β1 on clusterin mRNA expression as determined by real-time PCR. Values were normalized against GAPDH, and results are expressed as molecules clusterin per molecules GAPDH together with the relative decrease in RNA levels in treated cells compared with control cells ($^*P < 0.015$; $^{**}P < 0.002$; $^{***}P < 0.001$). (B) Effect of 1 ng/mL TGF-β1 on clusterin protein expression as determined by Western blot analysis of cell culture supernatants. Intensities of specific immunoreactive bands were quantified by computerized densitometry. Signal intensity of untreated control cells was set to 100%, and values represent the percentage expression of clusterin in treated cells compared with control cells, together with the relative decrease in protein levels ($^*P < 0.03$; $^{**}P < 0.001$; $^{***}P < 0.006$).
Ocular clusterin has been functionally implicated primarily in retinal (patho)physiology; increased expression has been shown in the retina of rd8 mutant mice,30 in the retina of rats after light-induced photoreceptor degeneration13 or ischemia and reperfusion injury,12 and in human patients with various retinal dystrophies (e.g., retinitis pigmentosa).11,35 Reduced expression of clusterin has been reported only in the keratinized conjunctival epithelium in severe ocular surface diseases, suggesting that this molecule is important for the maintenance of the ocular surface epithelium.35

The present study provides a comprehensive expression profile of clusterin in the adult human eye and demonstrates a nearly ubiquitous expression in the cornea, conjunctiva, trabecular meshwork, iris, lens, ciliary body, retina, and choroid, underscoring its fundamental biologic importance. Quantitative real-time PCR indicated high basal expression levels in all ocular tissues, most prominently in the ciliary processes, with an expression level 3 to 15 times higher than in other ocular tissues, pointing to their role as the main secretory site for clusterin in the anterior segment. On the protein level, clusterin was located primarily extracellularly in epithelial and vascular basement membranes and in stromal elastic fibers, which is consistent with its role as a secreted extracellular chaperone.

Our data further provide evidence for a significant down-regulation of clusterin mRNA in all anterior segment tissues of PEX eyes without and with glaucoma compared with normal and glaucomatous control eyes without PEX. This down-regulation was limited to anterior segment tissues of PEX eyes and was found to be independent of the presence or type of glaucoma. Additionally, in patients with clinically unilateral PEX syndrome, clusterin expression was found to be reduced in the affected eyes and normal in the unaffected fellow eyes. Accordingly, aqueous levels of clusterin were slightly but significantly increased in patients with PEX glaucoma in spite of reduced mRNA expression in anterior segment tissues. This increase may be explained by a passive influx of clusterin from the blood, where the average clusterin concentration is much higher (325 ± 100 μg/mL) than in aqueous humor,7 through an impaired blood–aqueous barrier, which is significantly more compromised in eyes of patients with PEX glaucoma than in other ocular tissues, pointing to their role as the main secretory site for clusterin in the anterior segment. On the protein level, clusterin was located primarily extracellularly in epithelial and vascular basement membranes and in stromal elastic fibers, which is consistent with its role as a secreted extracellular chaperone.

Clusterin has been shown to be strongly upregulated by various stress conditions, including hypoxia/ischemia and oxidative stress.15,16,20 In view of the pronounced oxidative stress and hypoxic conditions in the anterior chamber of PEX eyes,5,6 the finding of clusterin downregulation in anterior segment tissues is unexpected. However, it further supports the notion that cellular protection mechanisms may be impaired in PEX eyes, as indicated by decreased concentrations of ascorbic acid and glutathione in aqueous humor and reduced expression of glutathione-S-transferase-1 in anterior segment tissues of PEX eyes.5,8,42

Clusterin gene expression appears to be responsive to a variety of cytokines, growth factors, and stress-inducing agents through many potential regulatory elements within the clusterin gene promoter region.27 The conserved Ap-1 site was identified as responsive to TGF-β1,45 which has been shown to be significantly elevated in the aqueous humor of patients with PEX and is considered a key mediator in the fibrotic PEX process. In fact, TGF-β1 has been shown to be one major regulator of clusterin gene expression on a cell-type-specific basis in vitro, downregulating clusterin mRNA in several cell types, such as smooth muscle cells and astrocytes.44,45 In this study, TGF-β1 induced a significant downregulation of clusterin message and protein in nonpigmented epithelial cells in vitro, suggesting that TGF-β1 may also downregulate clusterin expression and secretion in PEX eyes in vivo.

The resultant deficiency in clusterin, a highly efficient extracellular chaperone, may, however, influence and promote the chronic, stable accumulation of PEX material, an abnormal fibrillar extracellular matrix product, in anterior segment tissues of PEX eyes. The characteristic PEX fibrils, which are composed of laterally aggregated microfibrillar subunits, contain predominantly epitopes of elastic fibers and microfibrils, such as elastin, amyloid P, vitronectin, fibrillin-1, and latent TGF-β-binding proteins (LTBP-1 and -2).2 Therefore, PEX syndrome has been described as a specific type of stress-induced elastosis, elastic microfibrillopathy, associated with the excessive production and abnormal aggregation of elastic microfibril components.

The present study showed generally reduced immunoreactivity for clusterin in ocular tissues of PEX eyes but prominent immunopositivity of PEX deposits proper, thereby describing a hitherto not identified component of PEX material. On the one hand, reduced immunoreactivity of clusterin was found in tissue areas of fibrosis and sclerosis, such as in patients with focal segmental glomerulosclerosis, together with markedly reduced serum and urine levels of clusterin.46 Accordingly, clusterin knock-out mice develop progressive glomerulosclerosis.47 On the other hand, prominent immunoreactivity for clusterin has been associated with a wide variety of abnormal extracellular deposits, such as drusen,17 corneal amyloid deposits,48 arteriosclerotic plaques,18 senile plaques in Alzheimer disease,19 and abnormal elastic fibers in chronic liver fibrosis.49 Taken together, these findings suggest that a deficiency in clusterin may promote tissue fibrosis and that clusterin may generally recognize misfolded and aggregated proteins in the extracellular space with high affinity.

Misfolding, aggregation, and precipitation of proteins are hallmarks of various chronic diseases generally classified as protein misfolding or conformational diseases (Alzheimer disease, Parkinson disease, Creutzfeldt-Jakob disease, amyloid-β, cataract, and diabetes mellitus type II).50 Misfolding of proteins results from structural modification, which is typically caused by mutation or oxidative injury, and dysfunction or overload of the proteasome system. In these diseases, clusterin participates in protein aggregation and associates with pathologic fibrinous deposits, such as amyloid plaques, in the extracellular space.19 In vitro, clusterin has been found to inhibit the formation of insoluble amyloid fibrils resulting from the aggregation of amyloid-β, apolipoprotein C-II, fragments of the prion protein, and a variety of other fibril-forming peptides.51,52 Clusterin has been also reported to associate with soluble amyloid-β in plasma and cerebrospinal fluid in vivo, suggesting that the interaction may preclude amyloid-β aggregation and fibrilization in biologic fluids.51,52 The association of clusterin with pathologic deposits in disease states may reflect the inability of this chaperone to completely inhibit the

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**Functional Significance of Clusterin Downregulation in PEX Syndrome**

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large-scale protein precipitation under pathologic conditions in which the precipitating species is present in excess.53,54

It is, therefore, conceivable that the oxidative milieu in the anterior chamber of PEX eyes and a dysfunction of the ubiquitin–proteasome system in PEX tissues may lead to stress-induced protein modifications and misfolding. The reduced expression and distinct deficiency of clusterin may further promote protein aggregation, leading to the chronic, stable accumulation of the characteristic pathologic material in anterior segment tissues of PEX eyes. However, whether PEX syndrome can be actually included in the category of protein aggregation diseases remains an open and challenging question.

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References


