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Increased Clusterin Expression in Fuchs' Endothelial Dystrophy

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Abstract

Purpose—To investigate the differential expression of the glycoprotein clusterin/apoJ (CLU) in normal and Fuchs' endothelial dystrophy (FED) corneal endothelium and to compare the expression of various forms of CLU in normal and FED tissue.

Methods—FED and pseudophakic bullous keratopathy (PBK) corneal buttons were removed during transplantation and normal corneas were obtained from tissue banks. Human corneal endothelial cells and Descemet's membrane (HCEC-DM) complex was dissected from the stroma. Proteins were separated on 2-D gels and subjected to comparative proteomic analysis. Relative expression of pre-secretory CLU (pre-sCLU), secretory CLU (sCLU), and nuclear CLU (nCLU) were compared between normal and FED HCEC-DM by Western blots. Expression of CLU mRNA was compared using RTPCR. Subcellular localization of CLU was compared in corneal whole mounts from normal and FED patients by immunocytochemistry followed by confocal microscopy.

Results—Proteomic analysis revealed an apparent increase in CLU expression in FED HCEC-DM compared with normal controls. Western blot analysis demonstrated that presCLU protein expression was 5.2 times higher in FED than in normal samples ($p=3.52E-05$), while the mature form modified for secretion (sCLU) was not significantly elevated ($p=0.092$). Expression of nCLU protein was significantly elevated in FED ($p=0.013$). RTPCR analysis revealed that CLU mRNA was significantly increased ($p=0.002$) in FED samples, but not in PBK samples. CLU had also a distinctive localization in FED samples with enhanced intracellular staining around the guttae and in the nuclei of endothelial cells.

Conclusion—CLU expression is markedly elevated in FED-affected tissue, pointing to a yet undiscovered form of dysregulation of endothelial cell function involved in FED pathogenesis.

Keywords

corneal endothelium; Fuchs' endothelial dystrophy; clusterin

Fuchs' endothelial corneal dystrophy (FED) is the most common endogenous cause of corneal endothelial dysfunction and leads to progressive corneal edema and blindness.¹ Currently, there is no effective cure for the disease due to the lack of therapeutic agents and the only modality available to restore vision is corneal transplantation. Recently, nuclear labeling and mRNA analysis techniques showed that apoptosis is involved in FED endothelial cell death.

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^{2–4} FED is characterized by extracellular collagenous deposits called “guttae” that accumulate posterior to Descemet’s membrane (DM)—mainly in the posterior banded layer, and subsequently thicken the DM.⁵ Ultrastructurally, the most commonly described DM abnormality in FED has been excessive and disorganized assembly of collagen VIII, an extracellular matrix molecule typically secreted by normal corneal endothelial cells during embryogenesis.^{6–8} Recently, early-onset FED, leading to corneal transplantation in the 4th decade, has been linked to a mutation in the COL8A2 gene and corresponding alterations in DM structures have been described.^{9, 10} The same genetic predisposition has not been elucidated in late-onset FED, a variant that is more common and accounts for the majority of FED cases.¹¹ Despite the identification of genetic factors that are linked with the early-onset disease, the pathophysiology of FED remains unclear. Specifically, the molecular basis for the formation of characteristic guttae and subsequent endothelial cell apoptosis is not well understood.

In this study, we hypothesized that there is a differential dysregulation of protein synthesis and/or secretion in FED-affected tissues that leads to abnormal extra cellular matrix (ECM) deposition and cellular apoptosis. In order to identify proteins that are differentially expressed in FED, 2-D gel analysis was performed and the profiles compared between proteins extracted from the endothelium-DM of diseased and healthy individuals. Following analysis, special focus was directed to protein spots at 30–40 kDa with isoelectric points ranging from 5.0 to 6.0. One series of spots within this area was identified by MALDI-TOF as clusterin.

Clusterin (CLU: also known as apolipoprotein J, testosterone-repressed prostate message-2, SP 40-40, complement lysis inhibitor, gp80, glycoprotein III, or sulphate glycoprotein-2) is a widely expressed heterodimeric disulfide-linked glycoprotein found in many tissues and body fluids.^{12–14} Expression of the CLU gene results in the synthesis of several different forms of CLU protein, located in different subcellular compartments. Following translation, CLU exists in an unglycosylated 60 kDa form, known as pre-secretory CLU (pre-sCLU). This form is targeted to the endoplasmic reticulum and subsequently to the Golgi where it is glycosylated and then proteolytically cleaved to form different, but similarly sized α - and β -subunits prior to secretion.^{15, 16} Mature, secreted clusterin (sCLU) is a 70–80 kDa glycosylated heterodimer, composed of both α - and β - subunits joined by disulfide bonds. sCLU appears on polyacrylamide gels as a 30–40 kDa protein smear under reducing conditions.¹⁷ The ability of sCLU amphipathic domains to bind hydrophobic molecules supports its role as a molecular chaperone in clearing cellular debris and scavenging denatured extracellular proteins.^{16, 18, 19} Secretory CLU is overexpressed in many tissues undergoing stress, including cancers and neurodegenerative disorders, and aids in cell survival under cytotoxic conditions.^{20–22} Due to its cytoprotective and anti-apoptotic properties, sCLU acts as a pro-survival factor for most cells.^{23, 24}

Several studies have demonstrated that there is another form of CLU, nuclear CLU (nCLU) that does not undergo α - and β -cleavage or extensive glycosylation.²⁵ This 49–55 kDa form has been shown to be located primarily in the nucleus. Although the exact role of nCLU is unclear, it is thought to bind Ku-proteins, which are involved in DNA repair.¹⁵ In response to stressors, such as ionizing radiation (IR) or conditions involving TGF- β upregulation, nCLU translocates to the nucleus, where it binds Ku-proteins, promoting apoptosis in stressed cells, hence acting as a pro-death protein.^{15, 25, 26} Although some authors propose that nCLU is synthesized from an alternatively spliced CLU mRNA, lacking exon II, there is no clear consensus as to whether nCLU and presCLU/ sCLU are the products of two different mRNA’s.^{27, 28} Nevertheless, multiple studies have shown that there are important functional differences in CLU forms based on their subcellular localization and that the differential identification of those forms can be reliably performed on the protein level by Western blotting and immunocytochemical studies.^{20, 27}

In the current study, we first employed 2-D gel electrophoresis to aid in protein “profiling”. Then, based on the findings, we investigated the relative expression of the various CLU forms in normal and FED-affected corneal endothelial cells on both the protein and mRNA levels. Immunocytochemistry was performed to compare cellular localization of CLU in normal and diseased tissues.

MATERIALS AND METHODS

Human Tissue

Donor confidentiality was maintained according to the Declaration of Helsinki. This study was approved by the Massachusetts Eye and Ear Institutional Review Board. Informed consent was obtained from patients undergoing corneal transplantation for Fuchs' endothelial dystrophy and pseudophakic bullous keratopathy (PBK). The FED and PBK corneal buttons were placed in Optisol-GS immediately after surgical removal and stored at 4°C prior to sample preparation (Table 1). Two-thirds of each FED and PBK corneal button were used for the study and one-third of the button was used for histopathological confirmation of the diagnosis. Normal human corneal buttons were obtained from the New England Tissue Bank (Boston, MA) and National Disease Research Interchange (Philadelphia, PA) and were used as controls. To assure donor tissue suitability, the current studies utilized exclusion criteria previously published from this laboratory.²⁹ Since normal corneal buttons were stored in Optisol-GS at 4°C prior to sample preparation, FED and PBK corneas were also stored in Optisol-GS 4°C to negate any effects of storage conditions on protein expression.

Sample Preparation

Table 1 presents information regarding the tissue samples used in these studies. For 2-D gel electrophoresis and Western blot analysis, samples were prepared by pooling protein extracts from two or more donors (Sample 1–5, Table 1) and by analyzing samples from individual donors (Samples 6 and 7, Table 1). For RT-PCR studies, samples from individual donors were analyzed, except for one pooled sample (Sample 11, Table 1). Samples 14–18, which were used for immunocytochemistry and RT-PCR, were from individual donors. Normal donors were decade-matched with FED and PBK donors. Corneal buttons were recovered from Optisol-GS and briefly rinsed in PBS. Under a dissecting microscope, Descemet's membrane along with the endothelial cell layer (HCEC-DM complex) was dissected from the stroma and washed with 10mM HEPES buffer (pH 7.4) before protein extraction. Samples used for 2-D gel electrophoresis were subjected to an additional washing step with HEPES buffer (10mM, pH 7.4) to reduce the concentration of salts. Protein extraction buffer ER3 (Bio-Rad, Hercules, CA, USA), containing 5 M urea, 2 M thiourea, 2% CHAPS, 2% SB 3–10, 40 mM Tris, 0.2% Bio-Lyte 3/10 ampholyte, and 1 mM tributyl phosphine (TBP), was added to the HCEC-DM sample. Proteins were solubilized by pipetting up and down to promote adequate mixing and then incubating the samples at room temperature for 30 minutes, followed by ultracentrifugation at 40,000 rpm, 21°C for 1 hour. HCEC-DM protein samples were used for 2D-gel electrophoresis and Western blot analysis. The protein concentration of the samples was determined by modified Bio-Rad protein assay.

2D-Gel Electrophoresis and Protein Identification

Equal amounts of protein from both the normal endothelium and FED samples (Table 1, Sample 1) were loaded onto immobilized, pH 3–10 linear gradient, 17 cm IPG strips (Bio-Rad, Hercules, CA, USA) for passive rehydration for 14 hrs. Isoelectric focusing was carried out using a Protean IEF Cell (Bio-Rad) with a gradual voltage increase up to 10,000 volts for a total of 60,000 volt-hours. Second-dimensional separation was performed using 8–16% pre-cast gradient polyacrylamide gels (BioRad). The gels (193×183×1.0 mm) were run at 350 volts until the bromophenol blue dye disappeared. Gels were then fixed in 10% methanol and 7%

acetic acid, stained overnight in SYPRO Ruby (Invitrogen, Carlsbad, CA), and washed in water for 1 hour before imaging. Protein spots from the 2D gel were imaged with a ProEXPRESS Proteomic Imaging System (PerkinElmer, Boston, MA) using optimized excitation (480/80) and emission (650/150) filters for SYPRO Ruby Protein Gel Stain. Of special interest was a series of protein spots at 30–40 kDa, with isoelectric points ranging from 5.0 to 6.0. Gel plugs from these spots were excised by direct picking using a ProXCISION spot-picking robot equipped with a CCD camera (PerkinElmer) and filter sets for Sypro Ruby. Gel pieces were placed in a ZipPlate (Millipore, Billerica, MA) and processed as described in the manufacturer's protocol. In brief, the gel plug was washed in 25 mM ammonium bicarbonate/5% acetonitrile for 30 minutes, and de-stained with ammonium bicarbonate/50% acetonitrile for 30 minutes $\times$ 2. Gel plugs were then dehydrated with 100% acetonitrile for 15 minutes, re-hydrated in 15 μ L of 25 mM ammonium bicarbonate containing 100 ng Trypsin Gold (Promega, Madison, WI), and then incubated at 30°C overnight. The C₁₈ resin of the ZipPlate was then activated with 9 μ L acetonitrile for 15 minutes at 37°C. Peptides were then washed out of the gel plug with 180 μ L 0.1% trifluoroacetic acid (TFA) for 30 minutes and then bound to the C₁₈ resin using low vacuum followed by washing twice with 100 μ L TFA under high vacuum. Peptides were then directly eluted onto a disposable MALDI target plate (PerkinElmer) by direct vacuum elution with matrix α -cyano-4-hydroxy cinnamic acid (LaserBiolabs, Sophid-Antipolis Cedex, France) (α -CHCA at 10mg/mL) in 50% acetonitrile/50% TFA. Matrix was allowed to air-dry allowing crystals to form. The MALDI plate was then loaded into a prO-TOF 2000 MALDI-TOF (PerkinElmer). The instrument was calibrated using a two-point calibration method. Sample data was acquired with a mass range of 750–4500 Da. Proteins were identified by searching a local copy of the NCBI (National Center for Biotechnology Information, www.ncbi.nih.gov) protein database using the ProFound search engine (Rockefeller University, New York, NY).

Western Blot Analysis

HCEC-DM samples from normal donors and FED patients (Table 1, Samples 2–7) were loaded on 10% Bis-Tris gels for SDS-PAGE. Peptides were then electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). Nonspecific binding was blocked by incubation for 1 hour at room temperature in 5% nonfat milk diluted in PBS. Membranes were incubated overnight at 4°C with rabbit polyclonal anti-clusterin (H-330) (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:400, and mouse monoclonal anti- β -actin (Sigma Aldrich, St. Louis, MO) diluted 1:6000 in blocking solution. Blots were rinsed, re-blocked, and exposed for 1 hour to horseradish peroxidase (HRP)-conjugated donkey anti-mouse IgG for β -actin and anti-rabbit IgG for clusterin. All secondary antibodies were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA) and diluted 1:2000 in blocking solution. After washing in 0.1% Triton X-100, peptides were detected with a pico chemiluminescent substrate (SuperSignal, Rockford, IL). Images were digitally scanned and analyzed with NIH Image software version 1.61 (available by ftp at <http://rsb.info.nih.gov/nih-image>). Protein was normalized according to β -actin content. Experiments were repeated at least two times. Results were averaged and the standard deviation (SD) calculated. Statistical analysis using Student's unpaired t-test was performed using Microsoft Excel 2002 for Windows XP (Redmond, Washington, USA). $P < 0.05$ was considered to be significant.

RT-PCR Detection of Clusterin mRNA

Total RNA was extracted from normal, FED, and PBK HCEC-DM complexes (Table 1, Samples 8–13, 17, 18) as recommended by the manufacturer (TRIzol; Invitrogen). RNA quantity and quality were assessed by spectrophotometric analysis. For all samples, cDNA was prepared by reverse transcription from equal amounts of RNA in a volume of 40 μ L using a commercially available kit (Promega). Table 2 provides information regarding the sequences

of the upstream and downstream primers used for RT-PCR and specific PCR conditions for CLU and β_2 -MG.^{27, 30} The PCR was performed in a 50 μ L reaction mixture containing equal amounts of normal, FED or PBK cDNA and 0.2 μ M of each of the upstream and downstream primers, plus reagents from a commercially available kit (Invitrogen). Specificity and yield of the PCR products were enhanced using the hot-start approach. The linear range of amplification reaction for CLU and β_2 -MG was tested using serial cDNA dilutions and by varying the number of cycles. For CLU, the PCR reaction was run for 25, 28, 30 and 35 cycles. At 35 cycles, the cDNA level was still within the exponential range. A total of 30 cycles was found to be optimal for β -MG. A 10-min extension was added at the end of all PCR. PCR products and 100-bp DNA ladder molecular weight markers were electrophoresed in 1.5% agarose gels containing 0.5 μ g/mL ethidium bromide and then photographed. β_2 -microglobulin (β_2 -MG) was used for normalization of cDNA load based on published papers^{30,32} and on personal communication with Dr. A.V. Ljubimov (Ophthalmology Research Laboratories, Cedars-Sinai Medical Center, Los Angeles, CA). Negative controls consisted of the PCR reaction mixture, including primers, but without cDNA. To ensure that the total RNA samples were not contaminated with genomic DNA, a negative control was used where cDNA of each sample was replaced by the same amount of total RNA in the PCR reaction mixture, along with 0.2 μ M each of β_2 -MG upstream and downstream primers. Images of PCR gels were obtained using an image-analysis system (Gel Doc 2000, BIO-RAD). Semiquantitative analysis of the images was made using NIH Image-J version 1.37v (<http://rsb.info.nih.gov/ij/download.html>). Experiments were repeated at least two times. Results were averaged and the standard deviation calculated. Statistical analysis using Student's unpaired t-test was performed using Microsoft Excel 2002 for Windows XP. $P < 0.05$ was considered to be significant. Specificity of the amplified CLU cDNA PCR product was confirmed by sequencing at the DNA Sequencing Center for Vision Research (DSCVR) at Massachusetts Eye and Ear Infirmary, Boston, MA.

Immunocytochemical Localization of Clusterin

Normal and FED corneas (Table 1, Samples 14–16) were washed in PBS and then fixed with 100% methanol for 10 minutes at -20°C . All subsequent steps were performed at room temperature. Corneas were washed 3 times in PBS for 10 minutes each, then permeabilized for 10 minutes with 1% Triton X-100 in PBS, and washed again 3 times in PBS for 10 minutes each. Nonspecific binding was blocked using 4% bovine serum albumin (BSA; Fisher, Pittsburgh, PA) in PBS for 10 minutes. Corneas were incubated for 2 hours in rabbit polyclonal anti-clusterin (H-330) diluted 1:50 in 4% BSA in PBS. Corneas were washed 3 times in PBS for 10 minutes each and then incubated for 1 hour with fluorescein (FITC)-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch) diluted 1:100 in 4% BSA in PBS. Negative controls consisted of secondary antibody alone. After being washed in PBS 3 times for 10 minutes each, corneas were placed endothelial-side up on slides using mounting medium containing propidium iodide for nuclear staining (Vector Laboratories, Burlingame, CA). Digital images were obtained using a Leica TSC-SP2 confocal microscope (Bannockburn, IL). A Z-series through the tissue was captured with a step size of 0.2 μ m per image. Images were created by using a single series or by collapsing Z-series images onto a single image plane by projecting the maximal pixel intensity of the images.

RESULTS

Differential CLU Expression in FED Corneas

To identify potential differences in protein expression between normal and FED human corneal endothelium, HCEC-DM samples were obtained from three FED patients and two decade-matched normal donors (Table 1, Sample 1). Comparison of the two protein patterns (Figure 1A, B) indicated that there were a number of similar protein spots; however, closer examination revealed a number of interesting differences. Among the patterns noted was a series of spots

migrating in the 30–40 kDa range with somewhat different isoelectric points (Figure 1C,D). MALDI-TOF identified each spot in this series as CLU. Although MALDI-TOF did not distinguish between these subunits, these CLU spots most likely correspond to the α - and β -subunits of the secreted form of CLU. The observed differences in isoelectric points most likely represent different post-translational modifications of these subunits. Comparison of the CLU spot patterns demonstrated the presence of a greater number of CLU spots in the FED sample compared with that of normal endothelium. As indicated above, gel electrophoresis normally reveals the presence of precursor forms of CLU at higher molecular weights; however, these higher molecular weight forms were not clearly distinguishable in the Sypro Ruby-stained 2D gels and, therefore, were not identified by MALDI-TOF. Within the 30–40 kDa region of the 2-D gels was an additional series of protein spots located above CLU. This series of spots, migrating at ~38 kDa, was identified as β IG-H3 protein. Although β IG-H3 appeared to show differences between the normal and FED gels, subsequent studies focused only on relative CLU expression. Differences in the relative expression of β IG-H3 will be investigated in a future study. β -actin (42 kDa) was also identified in both the normal and FED gels.

Western Blot Characterization of CLU Forms in Normal and FED Samples

Western blot analysis was performed to further characterize and compare the expression of the various forms of CLU in HCEC-DM samples from normal and FED donors. Western blotting was performed on a total of 4 pooled normal and 4 pooled FED samples (Samples 2–5, Table 1), and on 2 individual samples (Samples 6 and 7, Table 1). The data obtained from the pooled and individual samples was identical. The patterns and expression levels of CLU were consistently reproducible and did not change between pooled and individual samples or with variable storage times of specimens in Optisol-GS. Figure 2A shows a representative blot from a pooled FED and normal sample, while Figure 2B presents the densitometric comparison. It was established previously that the polyclonal anti-clusterin antibody (H-330) used for this analysis is able to detect all forms of CLU.³³ Molecular weights of the different CLU-positive bands observed on the Western blots closely corresponded with previously published Western blot data by Pucci et al.²⁰ A protein band, migrating at approximately 60 kDa, was identified as the precursor form of soluble CLU (pre-sCLU). Densitometric analysis indicated that pre-sCLU was expressed an average of 5.2-fold higher in the FED samples than in normal controls ($p=3.52E-05$). A band migrating at approximately 49 kDa was consistently observed in the FED-affected cells, but yielded only a very faint band or no band in normal controls. This band has been shown to correspond to the nuclear form of CLU (nCLU) in lysates from several different cell types. The average density of the nCLU band was 23.7-fold higher in FED samples than in samples from normal donors ($p=0.013$). The 30–40 kDa form of CLU corresponding to the alpha and beta chains of soluble CLU tended to be expressed at higher levels in FED samples, but did not show a statistically significant difference ($p=0.092$).

RT-PCR Detection of Clusterin mRNA

The mRNA expression of CLU was also compared between normal and FED samples using β_2 -microglobulin for normalization. As shown in Figure 3A, a single band corresponding to the expected weight of the CLU PCR product was observed in both the normal and FED samples. Densitometric analysis of the PCR products is shown in Figure 3B. Results indicate that the mRNA level of CLU was on average two-fold higher in FED tissue than in normal controls ($p=0.002$). The mRNA expression differences between normal and FED specimens were not affected by sample pooling or by the variable preservation time in Optisol-GS. Sequencing of the product confirmed that the PCR transcript was CLU. In order to evaluate whether CLU over-production is specific to FED corneas, mRNA expression of CLU was compared between normal and PBK samples using β_2 -MG for normalization. The comparison was performed between two sets of age-matched samples (Sample 17 and 18, Table 1). Relative CLU mRNA expression was lower in PBK HCEC-DM than in normal samples (Figure 3C).

Immunocytochemical Localization of Clusterin

Indirect immunofluorescence studies were performed to compare the localization of CLU in the endothelium of normal and FED donors. Corneal buttons from normal and FED donors were treated with the polyclonal clusterin antibody (H-330) known to be reactive against all forms of CLU.³³ Figure 4 presents confocal images in which the Z-series was collapsed onto a single image plane. Figure 4A – D presents confocal images of normal endothelium. In normal tissue, a relatively uniform, punctate distribution of CLU was observed within the cytoplasm (Figure 4A, C). Negative controls consisted of normal corneas incubated with secondary antibody only. No CLU-positive staining was observed under these conditions, indicating the specificity of primary antibody staining (Figure 4D). Interestingly, the CLU staining pattern in FED endothelium (Figure 4E – H) was quite different from that of normal HCEC. In FED-affected corneas, there was a rosette-type clustering of endothelial cells around dark areas containing no CLU- or PI-positive staining. Since the dark areas did not contain nuclei, they were considered to represent corneal guttae. CLU staining was present in a fine punctate pattern throughout the cytoplasm (Figure 4E, G, H). The centers of the guttae appeared to have some CLU-positive staining, but no nuclear staining was observed (Figure 4H), suggesting the presence of cell debris in these areas. To explore CLU localization in the nucleus, single Z-plane images taken through the nuclei of FED and normal endothelium were examined (Figure 5). HCEC in the FED specimens consistently showed increased staining for CLU in the nucleus compared with that in normal corneas (compare Figure 5A and D). In addition, the relative intensity of CLU staining in the cytoplasm of FED cells appeared increased compared with that in HCEC from normal donors.

Discussion

CLU is a ubiquitous glycoprotein that is especially abundant in cells at tissue-fluid interfaces and has been implicated in the maintenance of normal cell-extracellular matrix interaction.^{34, 35} Previous studies showed the presence of CLU in healthy human corneal endothelium.^{36, 37} In this study, the proteomic analysis of normal and FED HCEC-DM complexes suggests that post-translational processing and expression of CLU differ in FED tissue. Subsequently, targeted studies were performed to investigate the differential expression of specific forms of CLU in normal and FED endothelium. As a result, both the pro-survival and pro-apoptosis forms of CLU were found to be over-expressed in FED cells. This up-regulated CLU synthesis points to an undiscovered form of dysregulation of endothelial function involved in FED pathogenesis.

Several techniques were employed to characterize the differential expression of CLU forms between normal and FED specimens. When profiling the differential protein expression between normal and FED specimens, one of the most striking differences was the expression of sCLU in the 30–40 kDa range. Even though other protein differences were noted, one of them being an increased number of β IG-H3 spots in FED samples, we postponed the investigation of those differences for subsequent studies and focused on CLU. The MALDI-TOF identification of a greater number of sCLU spots in FED samples within the 30–40 kDa molecular weight range indicated that there were marked differences in the post-translational modification of sCLU in FED versus normal samples.

Western blot analysis further investigated CLU protein expression in both normal and FED endothelium. Expression of CLU in FED HCEC was significantly higher than in normal HCEC for both the nuclear and pre-secretory forms. Of interest was the finding that the level of the 30–40 kDa sCLU, which is the secreted form of CLU, was not significantly elevated in FED cells. A study by O'Sullivan et al.²⁸ noted that, in MCF-7 epithelial cells, the proteolytic cleavage required to produce the mature secretory form of CLU occurs in the Golgi prior to extracellular secretion. Stressing the cells with pro-apoptotic stimuli, such as TNF- α , blocked

the proteolysis of pre-sCLU in the Golgi and prevented the formation of the secreted α and β chains. Therefore, it is possible, that FED cells have an alteration in the post-translational modification of pre-sCLU, preventing a parallel increase in pre-sCLU and sCLU. Similarly, Nizard et al.³⁸ showed that, under certain stressed conditions, CLU can evade the secretion pathway altogether and localize mainly within the cytosol, where it exerts its biological functions. Separate studies have shown that the intracellular 60 kDa form of CLU, and not the secretory 40 kDa isoform, is responsible for the anti-apoptotic effects of CLU by interfering with Bax activation in mitochondria.³⁹

FED-affected endothelial cells were also found to have elevated levels of nCLU as compared to the normal cells. The nuclear CLU 49 kDa band was consistently present in FED, but not in normal cells by Western blotting. These elevated levels of nCLU correlated with increased nuclear staining of CLU in FED-affected cells by confocal microscopy, indicating that increased production of nCLU is followed by its translocation to the nucleus in the pathological state, but not in the normal cells. These data are in agreement with previous studies that showed induction and translocation of CLU from the cytoplasm to the nucleus after cytotoxic stimulation with IR and TGF- β treatment.^{25, 27} Separate studies have shown that over-expression of nCLU without cytotoxic stimulation leads to cell death, pointing out its role in apoptosis independent of exogenous causes.^{15, 40} In the nucleus, nCLU has been shown to interact with the Ku70 subunit of the Ku70/80 protein, which is involved in DNA double-strand break repair.^{15, 17, 27} When bound to the over-expressed nCLU, Ku70/80 is prevented from DNA end-binding, thus preventing repair of genomic breaks and leading to genomic instability. Although additional information is needed regarding how nCLU affects the DNA repair process, it is known, that over-expression of nCLU causes diminished cell growth and leads to lethality.¹⁵

To investigate whether the mRNA level of CLU increases in FED cells, RT-PCR analysis was performed. We used a well-established primer set, which amplifies all four CLU exons and detects the full length form of CLU.²⁷ There was a two-fold increase in CLU cDNA in FED cells vs. normals, indicating that there is an overall increase in CLU mRNA, as well as protein, expression in the diseased cells. The RT-PCR and Western blot data was identical regardless of storage time and the use of single or pooled samples. In additional experiments (data not shown), we used a primer set reported to specifically amplify the nuclear form of CLU²⁷, but could not obtain consistent results—a finding similar to that of other investigators (personal communication with Dr. Denis Michel, Université de Rennes, Rennes, France).³⁸ The mRNA analysis of the tissue taken from the pseudopapillary keratopathy specimens revealed a relative decrease in CLU production as compared to normal specimens, indicating that a similar increase in CLU mRNA expression does not occur in PBK. Such findings indicate that CLU overexpression in FED may be specific to the pathogenesis of the dystrophy and not seen under other corneal swelling conditions.

Confocal microscopy revealed an unusual CLU staining pattern in the FED endothelium. CLU exhibited mostly intracellular staining, which was highlighted at the edges of the cell membranes next to dark circular areas suggestive of guttae. The central fluorescence within those dark areas could represent CLU expression in the remnants of dying cells. It is also possible that the unusual staining pattern in those areas signifies the propensity of CLU to associate with dead cells lacking intact cell membranes, as shown to occur in L929-pRc.clus cells in response to TNF- α stimulation.⁴¹ The physiological relevance of such an interaction is not known.

In FED tissue endothelial cell nuclei clustered densely around the guttae, and those cells had an enhanced CLU staining at the cell membrane borders next to guttae. Such a staining pattern most likely represents CLU's essential role in eliciting endothelial cell clustering under stressed

conditions. Previous studies showed that CLU induced cell aggregation in response to oxidant injury due to hydrogen peroxide. The resultant CLU-induced cell aggregation was shown to protect the cells against injury by decreasing the amount of cell membrane accessible to oxidant injury and by maintaining better cell to cell contacts, that, when disrupted, can lead to apoptosis.^{42, 43}

The finding that CLU is over-expressed in FED appears to be important in elucidating its pathophysiology. Over-expression of pre-sCLU may be a stress-induced response to protect the cells from apoptosis. Numerous studies have shown that levels of CLU are often elevated in response to a variety of tissue insults.^{24, 44, 45} The prevailing thought is that CLU can act as an intracellular and extracellular chaperone and protect a variety of proteins from stress-induced precipitation by affecting their folding state.^{18,19} CLU has been shown to play a role in protection of kidney from ischemic glomerular injury^{46, 47}; and cancer cells from apoptosis induced by chemotherapeutic agents.^{39,45} CLU is also over-expressed in many pathologic conditions, two of which are Alzheimer's disease (AD) and age-related macular degeneration (ARMD).^{21, 48} Similar to FED, both of these disorders manifest with high amounts of extracellular membrane deposits (i.e. drusen and amyloid plaques) and concomitant dysfunction and apoptosis of the cells next to the deposits. Initial studies of AD showed that CLU protects neurons from amyloid plaque formation in vitro^{47, 49}, however, in a mouse model of AD, CLU promoted amyloid plaque accumulation and neuron toxicity.⁵⁰ Similarly, in the ARMD model, large amounts of CLU found in drusen were thought to promote the formation of these β -amyloid-like deposits.⁵¹ Although the exact function of CLU is not clear, the findings of CLU dysregulation in numerous pathological states point to a potentially common downstream pathway in these processes. Most studies do arrive at the same consensus though, and that is that CLU's chaperone-like properties may induce alterations in the equilibrium between the deposited and cleared material.⁵⁰

The relationship between the levels of sCLU and nCLU is not completely understood, especially how it can promote and inhibit cell death depending on the isoform expression. In colorectal carcinoma, there is a diminished expression of nCLU and increasing expression of sCLU with increasing cancer grade.²⁰ Other studies have shown that pro-apoptotic stimuli, like IR, increase the levels of both nCLU and pre- and sCLU proteins, the latter two forms showing a much higher increase than the former.^{15, 52} In the IR model, pre-sCLU and sCLU levels increased with low, nontoxic, growth stimulatory levels of IR, and nCLU levels increased with much higher levels of the cytotoxic stress.¹⁵ Similarly to the IR-induced CLU overexpression, both pre-secretory and nuclear isoforms were elevated in FED. Although the driving force for CLU production in FED is yet to be elucidated, one of the potential factors might be oxidative stress. Numerous studies have shown that oxidative stress and reactive oxygen species can induce CLU overproduction and that CLU can render the cells resistant to reactive oxygen species-mediated cellular injury.^{24, 42, 53} There is mounting evidence in the current literature that oxidative stress plays a role in FED.^{54, 55} Therefore, it is possible that dysregulation of CLU production is indirectly pointing to the mechanism of FED pathogenesis involving oxidative stress-induced damage to the corneal endothelium.

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Figure 1.

Sypro Ruby-stained 2-dimensional gels of human corneal endothelial cell - Descemet's membrane (HCEC-DM) proteins from normal (A) and Fuchs' endothelial dystrophy (FED) donors (B). Boxes in (A) and (B) show protein spots that were identified by MALDI-TOF. An enlarged view of these areas is shown in (C) for the normal donor sample and in (D) for the FED sample. Proteins were identified as Clusterin (CLU), β IGH3 (ellipses), and β -actin (arrows).

Figure 2.

Comparative analysis of clusterin (CLU) protein expression in HCEC-DM extracts from FED and normal donors. **(A)** Representative Western blot comparing CLU protein expression in extracts from FED and normal donors. Pre-sCLU is the uncleaved, precursor form of secreted clusterin (60 kDa); nCLU is the uncleaved and nonglycosylated form of CLU that is targeted for the nucleus (49–55 kDa); and sCLU indicates the α - and β - subunits of the mature, secreted form of CLU (30–40 kDa). beta actin was used for normalization of protein load. **(B)** Averaged data showing relative expression of three CLU forms from FED and normal samples (Table 1, Samples 2–7). Bars: SD. * $P < 0.05$. n.s.: not statistically significant.

Figure 3.

Comparative analysis of clusterin (CLU) mRNA expression in HCEC-DM extracts from FED, pseudophakic bullous keratopathy (PBK) and normal controls. **(A)** Representative ethidium bromide-stained gels demonstrating PCR products of the expected size for CLU and β_2 -microglobulin (β_2 -MG). On the left are cDNA markers in 100 bp increments with the bottom band at 100 bp. C(-): Negative control sample containing all reagents except cDNA; FED: cDNA from a 41 year old FED donor; N: cDNA from a 53 year old normal donor; C(+): cDNA from SW480 cells used as a positive control. **(B)** Densitometric comparison of the average amount of cDNA product for CLU primer from normal and FED samples (Table 1, Samples 8–13). β_2 -MG was used for normalization. Bars: SD. * $P < 0.05$. **(C)** Representative gels

demonstrating PCR products of the expected size for CLU and β_2 -MG. C(-): Negative control sample containing all reagents except cDNA; PBK: cDNA from a PBK donor; N: cDNA from a normal donor.

Figure 4.

Representative confocal images of normal (A–D) and FED endothelium (E–H) in whole-mounts of corneal tissue. Uniform, punctate clusterin (CLU) staining (green) was present in normal corneal endothelium (A). Image in (B) shows propidium iodide staining of nuclei (red) in the same tissue, while the image in (C) shows an overlay of the two images. Negative control, incubated in secondary antibody only, shows no discernable staining for CLU (D). In (E), the distribution of CLU staining in FED-affected cells forms a rosette pattern around the dark areas that represent corneal guttae (asterisks). Image in (F) shows PI-stained nuclei in the same tissue. Image in (G) is an overlay of the two images. Magnified view of FED endothelium (H) shows a clustering of CLU-positive cells around a gutta and some positive CLU staining not associated

with nuclei in the center of the gutta (arrow), suggesting the presence of cellular debris. Final magnification in **(A–G)**: 400X with 4 zoom. Final magnification in **(H)**: 400X with 8 zoom.

Figure 5.

Representative confocal images taken through a single, 0.2 micron-thick Z-plane of normal (A–C) and FED (D–F) corneal endothelium in whole mounts of corneal tissue. Images were taken after immunostaining for clusterin (CLU) (green: A and C) and PI-staining of nuclei (red: B and E). Comparison of overlayed images in (C) and (F) indicate the presence of CLU within nuclei. The punctate CLU staining pattern in the nuclei of FED cells (D) appears to be more concentrated than the CLU staining in the nuclei of cells from normal donors (A). Nuclear images taken immediately above and below showed similar staining patterns and indicate that the images were taken within the center of the nuclei. Final magnification: 400X with 8 zoom.

Table 1

Donor Information

Pooled sample #	FED HCEC-DM†		Normal HCEC-DM and/or stroma/epithelium		Use of Samples		
	Age	Sex	Time in Optisol, (days)	Age	Sex	Death-to-Preservation, (hours)*	Time in Optisol, (days)
1	64 72 77	F F F	10 10 3	77 78	M M	5.8 17	10 12
2	59 67	F F	8 1	53 64	F F	19 19	14 8
3	85 73 49 77 62	M M F M F	2 1 1 6 1	52 64 80	M F M	20 23 6	14 14 1
4	81 67 69	M M M	9 1 1	72 67	M M	10 11	14 15
5	69 66	M F	3 7	72 67	F F	11 11	14 15
6	75	F	1	73	M	4	3
7	55	F	1	68	M	7	1
8	41	F	1	53	M	4	1
9	57	F	6	56	M	8	8
10	82	F	7	72	F	15	6
11	78	F	4	77 84	F M	11 10	7 5
12	77	M	6	72	M	9	6
13	63	M	1	66	M	12	1
14	46	M	2	49	M	21	8
15	83	M	1	69	M	15	6
16	64	F	1	65	F	5	2
PBK HCEC-DM		Normal HCEC-DM					
17	70	M	1	70	F	8	16

Pooled sample #	FED HCEC-DM [†]		Normal HCEC-DM and/or stroma/epithelium			Use of Samples	
	Age	Sex	Time in Optisol, (days)	Age	Sex	Death-to-Preservation*, (hours)	Time in Optisol, (days)
18	44	M	1	53	M	4	1

FED: Fuchs' endothelial dystrophy
HCEC-DM: Human corneal endothelium and Descemet's membrane
PBK: Pseudophakic bullous keratopathy
[†]FED specimens taken during keratoplasty were placed in Optisol-GS at 4°C immediately.
*Time (hours) between death and placement of the cornea in Optisol-GS at 4°C.
**Time (days) from preservation in Optisol-GS to experimental use.

Table 2

Oligonucleotide Primer Sequences and PCR Conditions.

Amplified fragments	Sense primers	Antisense primers	PCR conditions
CLU 340 bp ²⁷	5'-ACAGGGTGCC GCTGACC-3'	5'-TTAGAGTCCTTC AGCTTTGTCTCTG-3'	94 °C for 30 s, 55°C for 30 s, 72°C for 45 s
β_2 -MG 335 bp ³⁰	5'-CTCGCGCTAC TCTCTCTTTCTG-3'	5'-GCTTACATGTCTC GATCCCACTT-3'	94 °C for 30 s, 50°C for 30 s, and 72°C for 45 s