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Increased Clusterin Expression in Fuchs' Endothelial Dystrophy

Ula V. Jurkunas,^{1,2,3} Maya S. Bitar,^{1,3} Ian Rawe,¹ Deshea L. Harris,^{1,3} Kathryn Colby,^{1,2,3} and Nancy C. Joyce^{1,3}

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 presecretory CLU (pre-sCLU), secretory (s)CLU, and nuclear (n)CLU were compared between normal and FED HCEC-DM by Western blot analysis. Expression of CLU mRNA was compared by using RT-PCR. Subcellular localization of CLU was compared in corneal wholemounts from normal eyes and eyes with FED by immunocytochemistry followed by confocal microscopy.

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

CONCLUSIONS. 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. (*Invest Ophthalmol Vis Sci.* 2008;49:2946-2955) DOI:10.1167/iov.07-1405

Fuchs' endothelial corneal dystrophy (FED) is the most common endogenous cause of corneal endothelial dysfunction and leads to progressive corneal edema and blindness.¹ Cur-

rently, 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.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 fourth 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 most 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 extracellular matrix (ECM) deposition and cellular apoptosis. To identify proteins that are differentially expressed in FED, we performed 2-D gel analysis, and compared the profiles between proteins extracted from the endothelium-DM of diseased and healthy individuals. After analysis, special focus was directed to protein spots at 30 to 40 kDa with the isoelectric point (pI) ranging from 5.0 to 6.0. One series of spots within this area was identified by MALDI-TOF (matrix-assisted desorption ionization-time of flight) mass spectrometry as clusterin (CLU).

CLU (also known as apolipoprotein J, testosterone-repressed prostate message-2, SP 40-40, complement lysis inhibitor, gp80, glycoprotein III, or sulfate 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. After translation, CLU exists in an unglycosylated 60-kDa form, known as presecretory 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 before secretion.^{15,16} Mature, secreted clusterin (sCLU) is a 70- to 80-kDa glycosylated heterodimer, composed of both α - and β -subunits joined by disulfide bonds. sCLU appears on polyacrylamide gels as a 30- to 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 those in cancers and neurodegenerative disorders, and aids in cell survival un-

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TABLE 1. Donor Information

| Pooled Sample | FED HCEC-DM* | | | Normal HCEC-DM and/or Stroma/Epithelium | | | | Use of Samples |
|---------------|--------------|-------------|---------------------|---|----------------|---------------------------------|----------------------|-----------------------|
| | Age | Sex | Time in Optisol (d) | Age | Sex | Death-to-Preservation, (hours)† | Time in Optisol (d)‡ | |
| 1 | 64 | F | 10 | 77 | M | 5.8 | 10 | 2-D gel |
| | 72 | F | 10 | 78 | M | 17 | 12 | |
| | 77 | F | 3 | | | | | |
| 2 | 59 | F | 8 | 53 | F | 19 | 14 | Western blot analysis |
| | 67 | F | 1 | 64 | F | 19 | 8 | |
| 3 | 85 | M | 2 | 52 | M | 20 | 14 | |
| | 73 | M | 1 | 64 | F | 23 | 14 | |
| | 49 | F | 1 | 80 | M | 6 | 1 | |
| 4 | 77 | M | 6 | | | | | |
| | 62 | F | 1 | | | | | |
| | 81 | M | 9 | 72 | M | 10 | 14 | |
| 5 | 67 | M | 1 | 67 | M | 11 | 15 | |
| | 69 | M | 1 | | | | | |
| | 69 | M | 3 | 72 | F | 11 | 14 | |
| 6 | 66 | F | 7 | 67 | F | 11 | 15 | |
| | 75 | F | 1 | 73 | M | 4 | 3 | |
| 7 | 55 | F | 1 | 68 | M | 7 | 1 | |
| 8 | 41 | F | 1 | 53 | M | 4 | 1 | RT-PCR |
| 9 | 57 | F | 6 | 56 | M | 8 | 8 | |
| 10 | 82 | F | 7 | 72 | F | 15 | 6 | |
| 11 | 78 | F | 4 | 77 | F | 11 | 7 | |
| | | | | 84 | M | 10 | 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 | Immunohistochemistry |
| 15 | 83 | M | 1 | 69 | M | 15 | 6 | |
| 16 | 64 | F | 1 | 65 | F | 5 | 2 | RT-PCR |
| | | | | | | | | |
| | | PBK HCEC-DM | | | Normal HCEC-DM | | | |
| 17 | 70 | M | 1 | 70 | F | 8 | 16 | |
| 18 | 44 | M | 1 | 53 | M | 4 | 1 | |

* 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.

der cytotoxic conditions.²⁰⁻²² Because of its cytoprotective and antiapoptotic properties, sCLU acts as a prosurvival 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- to 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 proapoptosis protein.^{15,25,26} Although some investigators have proposed that nCLU is synthesized from an alternatively spliced CLU mRNA, lacking exon II, there is no clear consensus as to whether nCLU and pre-sCLU/sCLU are the products of two different mRNAs.^{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 blot analysis and immunocytochemical studies.^{20,27}

In the present study, we first used a two-dimensional (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 FED and pseudophakic bullous keratopathy (PBK). The FED and PBK corneal buttons were placed in corneal preservative (Optisol-GS; Bausch & Lomb, Rochester, NY) immediately after surgical removal and stored at 4°C before sample preparation (Table 1). Two-thirds of each FED and PBK corneal button was used for the study and one-third of the button was used for histopathologic 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 control corneas. To assure donor tissue suitability, the current studies used exclusion criteria previously published from this laboratory.²⁹ Since normal corneal buttons were stored in preservative at 4°C before sample preparation, FED and PBK corneas were also

TABLE 2. Oligonucleotide Primer Sequences and PCR Conditions

| Amplified Fragments | Sense Primers | Antisense Primers | PCR Conditions |
|------------------------------------|------------------------------|----------------------------------|---|
| CLU 340 bp ²⁷ | 5'-ACAGGGTGCCGCTGACC-3' | 5'-TTAGAGCTCCTTCAGCTTTGTCTCTG-3' | 94°C for 30 s, 55°C for 30 s, 72°C for 45 s |
| β_2 -MG 335 bp ³⁰ | 5'-CTCGCGCTACTCTCTCTTTCTG-3' | 5'-GCTTACATGTCTCGATGCCACTT-3' | 94°C for 30 s, 50°C for 30 s, and 72°C for 45 s |

stored in preservative at 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 (Table 1, samples 1-5) and by analyzing samples from individual donors (Table 1, samples 6 and 7). For RT-PCR studies, samples from individual donors were analyzed, except for one pooled sample (Table 1, sample 11). Samples 14 to 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 preservative 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 10 mM 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 (10 mM, pH 7.4) to reduce the concentration of salts. Protein extraction buffer ER3 (Bio-Rad, Hercules, CA), containing 5 M urea, 2 M thiourea, 2% CHAPS, 2% SB 3-10, 40 mM Tris, 0.2% 3:10 ampholyte (Bio-Lyte; Bio-Rad), 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 a modified Bio-Rad protein assay.

2-D 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 to 10 linear gradient, 17-cm IPG strips (Bio-Rad) for passive rehydration for 14 hours. Isoelectric focusing was performed with an IEF cell (Proteom; 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% to 16% precast gradient polyacrylamide gels (Bio-Rad). 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 (Sypro Ruby Protein Gel Stain; Invitrogen, Carlsbad, CA), and washed in water for 1 hour before imaging. Protein spots from the 2-D gel were imaged (ProExpress Proteomic Imaging System; PerkinElmer, Boston, MA) using optimized excitation (480/80) and emission (650/150) filters for the stain. Of special interest was a series of protein spots at 30 to 40 kDa, with pI ranging from 5.0 to 6.0. Gel plugs from these spots were excised by direct picking using a spot-picking robot equipped with a charge-coupled device (CCD) camera (ProXcision; PerkinElmer) and filter sets for the stain. Gel pieces were placed in a plate (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 destained with ammonium bicarbonate/50% acetonitrile twice for 30 minutes each time. Gel plugs were then dehydrated with 100% acetonitrile for 15 minutes, rehydrated in 15 μ L of 25 mM ammonium bicarbonate containing 100 ng

mass-spectrometry grade trypsin (Trypsin Gold; Promega, Madison, WI) and then incubated at 30°C overnight. The C₁₈ resin of the plate (ZipPlate; Millipore) 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 (matrix assisted laser desorption ionization) target plate (PerkinElmer) by direct vacuum elution with matrix α -cyano-4-hydroxy cinnamic acid (α -CHCA at 10 mg/mL; LaserBiolabs, Sophid-Antipolis, France) in 50% acetonitrile/50% TFA. Matrix was air dried, allowing crystals to form. The MALDI plate was then loaded into a MALDI-TOF system (pro-TOF 2000; PerkinElmer). The instrument was calibrated with a two-point calibration method. Sample data were acquired with a mass range of 750 to 4500 Da. Proteins were identified by searching a local copy of the NCBI (provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD; 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). 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-CLU (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, reblocked, and exposed for 1 hour to horseradish peroxidase (HRP)-conjugated donkey anti-mouse IgG for β -actin and anti-rabbit IgG for CLU. All secondary antibodies were obtained from Jackson Immuno-Research Laboratories, Inc. (West Grove, PA) and diluted 1:2000 in blocking solution. After they were washed in 0.1% Triton X-100, the peptides were detected with a chemiluminescent substrate (Pico SuperSignal; Pierce Biotechnology, Rockford, IL). Images were digitally scanned and analyzed with NIH Image software, version 1.61 (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD, 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. The results were averaged and the standard deviation calculated. Statistical analysis was performed with Student's unpaired *t*-test (Excel 2002 for Windows XP; Microsoft, Redmond, WA). *P* < 0.05 was considered to be significant.

RT-PCR Detection of CLU 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 (β_2 -microglobulin).^{27,30} The PCR was performed in a 50- μ L reaction mixture containing equal amounts of normal, FED, or PBK

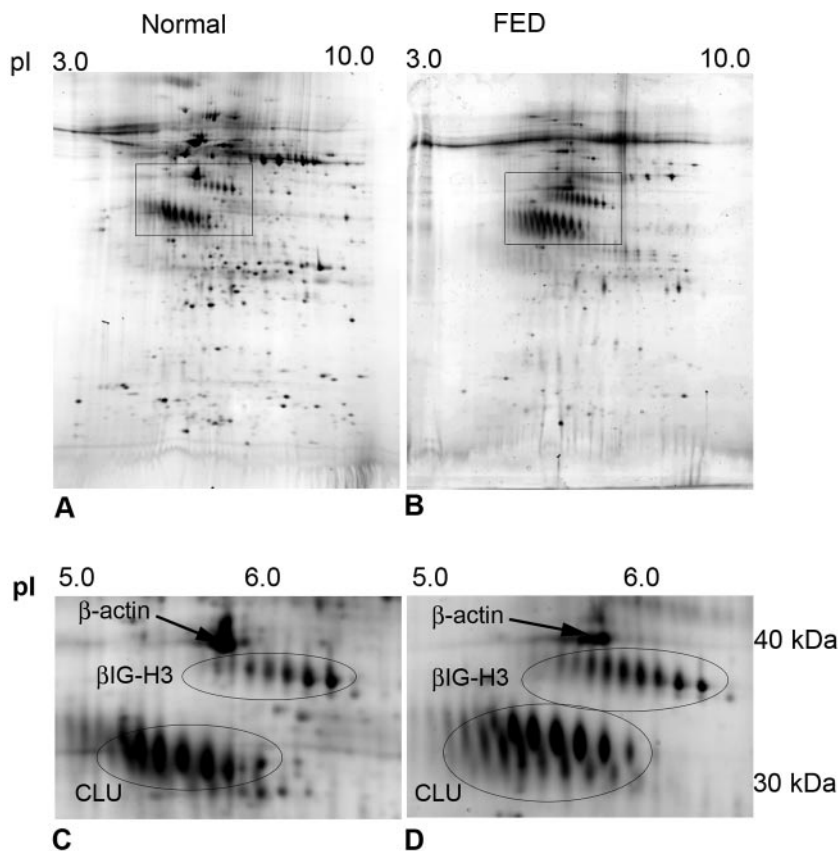


FIGURE 1. 2-D gels (Spyro Ruby stain; Invitrogen) of HCEC-DM proteins from normal (A) and FED donors (B). Boxes show protein spots that were identified by MALDI-TOF. An enlarged view of these areas is shown in (C) for the normal donor sample and in (D) for the FED sample. Proteins were identified as CLU, β IG-H3 (*ellipses*), and β -actin (*arrows*).

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 by using the hot-start approach.³¹ The linear range of amplification reaction for CLU and β 2-MG was tested by 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. Thirty cycles was found to be optimal for β 2-MG. A 10-minute extension was added at the end of all PCRs. 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-MG was used for normalization of cDNA load based on published papers^{30,32} and on personal communication (2007) with Alexander V. Ljubimov (Ophthalmology Research Laboratories, Cedars-Sinai Medical Center, Los Angeles, CA). Negative control experiments 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 with 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>). The experiments were repeated at least two times. The results were averaged and the standard deviation calculated. Statistical analysis was performed with Student's unpaired *t*-test (Excel 2002 for Windows XP; Microsoft). $P < 0.05$ was considered to be significant. The 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.

Immunocytochemical Localization of CLU

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 three times in PBS for 10 minutes each, then permeabilized for 10 minutes with 1% Triton X-100 in PBS, and washed again three times in PBS for 10 minutes each. Nonspecific binding was blocked using 4% bovine serum albumin (BSA; Fisher Scientific, Pittsburgh, PA) in PBS for 10 minutes. Corneas were incubated for 2 hours in rabbit polyclonal anti-CLU (H-330) diluted 1:50 in 4% BSA in PBS. The corneas were washed three 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 three times for 10 minutes each, corneas were placed endothelial-side up on slides in mounting medium containing PI for nuclear staining (Vector Laboratories, Burlingame, CA). Digital images were obtained with a confocal microscope (TSC-SP2; Leica, 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 maximum 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 (Figs. 1A, 1B) indicated that there were several similar protein spots; however, closer examination revealed several interesting differences. Among the patterns noted was a series of spots migrating in the 30- to 40-kDa range with a somewhat different pI (Figs. 1C, 1D). MALDI-TOF identified each spot in this series as CLU. Although MALDI-TOF did not distinguish between the subunits, these CLU spots

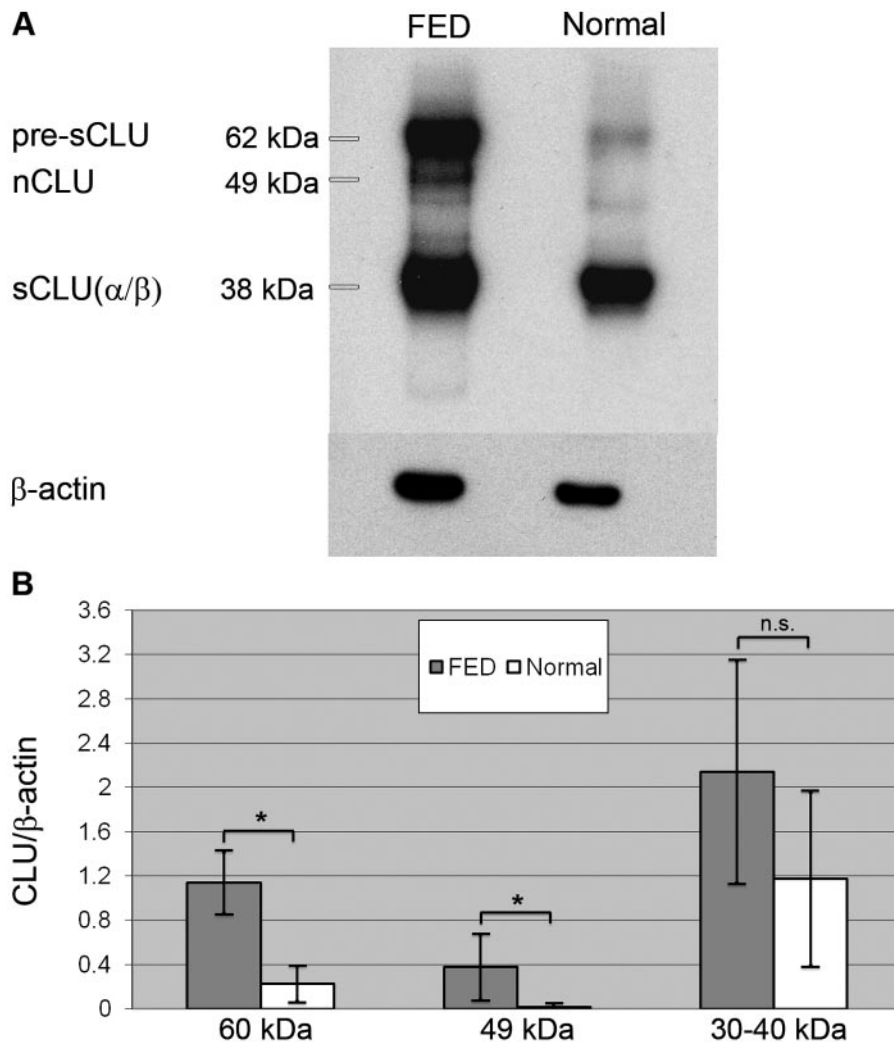


FIGURE 2. Comparative analysis of protein expression in HCEC-DM extracts from FED and normal donors. **(A)** Representative Western blot comparing CLU protein expression in extracts from FED and normal donors. Pre-sCLU is the uncleaved, precursor form of secreted clusterin (60 kDa); nCLU is the uncleaved 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). β -Actin was used to normalize protein loading. **(B)** Averaged data showing relative expression of three CLU forms from FED and normal samples (Table 1, samples 2–7). Bars, SD; * $P < 0.05$. NS, not statistically significant.

most likely correspond to the α - and β -subunits of the secreted form of CLU. The observed differences in pI most likely represent different posttranslational 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 earlier, 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 stained 2D gels (Sypro Ruby stain; Invitrogen) and, therefore, were not identified by MALDI-TOF. Within the 30- to 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 characterize further and compare the expression of the various forms of CLU in HCEC-DM samples from normal and FED donors. Western blot analysis was performed on four pooled normal and four pooled FED samples (Table 1, samples 2–5), and on two individual

samples (Table 1, samples 6 and 7). The data obtained from the pooled and individual samples were 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 preservative (Optisol-GS; Bausch & Lomb). Figure 2A shows a representative blot from a pooled FED and normal sample, and Figure 2B presents the densitometric comparison. It was established previously that the polyclonal anti-CLU 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 blot analysis 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- to 40-kDa form of CLU corresponding to the α and β chains of soluble CLU tended to be expressed at higher levels in FED samples, but did not show a statistically significant difference ($P = 0.092$).

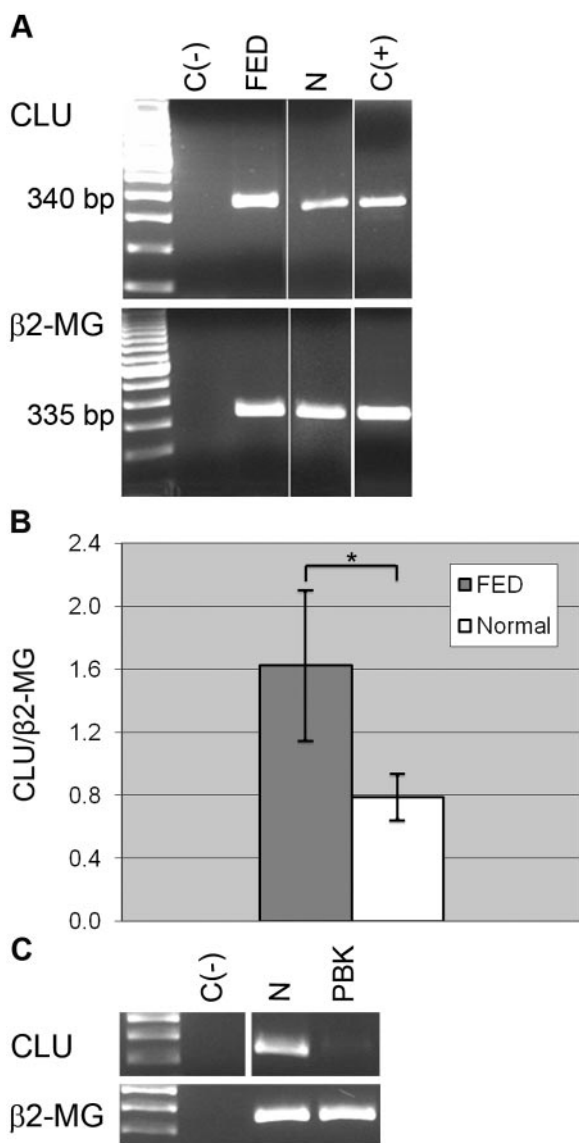


FIGURE 3. Comparative analysis of CLU mRNA expression in HCEC-DM extracts from FED, PBK, and normal control corneas. (A) Representative ethidium bromide-stained gels demonstrating PCR products of the expected size for CLU and β 2-MG. On the left are cDNA markers in 100-bp increments with the bottom band 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.

RT-PCR Detection of CLU mRNA

The mRNA expression of CLU was also compared between normal and FED samples using β 2-MG 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, twofold higher in FED tissue than in the normal control ($P = 0.002$). The mRNA expression

differences between normal and FED specimens were not affected by sample pooling or by the variable time in preservative. Sequencing of the product confirmed that the PCR transcript was CLU. To evaluate whether CLU overproduction 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 (Table 1, samples 17 and 18). Relative CLU mRNA expression was lower in PBK HCEC-DM than in normal samples (Fig. 3C).

Immunocytochemical Localization of CLU

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 CLU 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. Figures 4A–D present confocal images of normal endothelium. In normal tissue, a relatively uniform, punctate distribution of CLU was observed within the cytoplasm (Figures 4A, 4C). 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 (Fig. 4D). Of interest, the CLU staining pattern in FED endothelium (Figs. 4E, 4H) was quite different from that of normal HCECs. 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 (Figs. 4E, 4G, 4H). The centers of the guttae appeared to have some CLU-positive staining, but no nuclear staining was observed (Fig. 4H), suggesting the presence of cell debris in these areas. To explore CLU localization in the nucleus, we examined single z -plane images taken through the nuclei of FED and normal endothelium (Fig. 5). HCECs in the FED specimens consistently showed increased staining for CLU in the nucleus compared with that in normal corneas (compare Figs. 5A, 5D). In addition, the relative intensity of CLU staining in the cytoplasm of FED cells appeared increased compared with that in HCECs of 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} Studies have shown 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 posttranslational 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 proapoptosis forms of CLU were found to be overexpressed in FED cells. This upregulated CLU synthesis points to an undiscovered form of dysregulation of endothelial function involved in FED pathogenesis.

Several techniques were used 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- to 40-kDa range. Even though other protein differences were noted, one

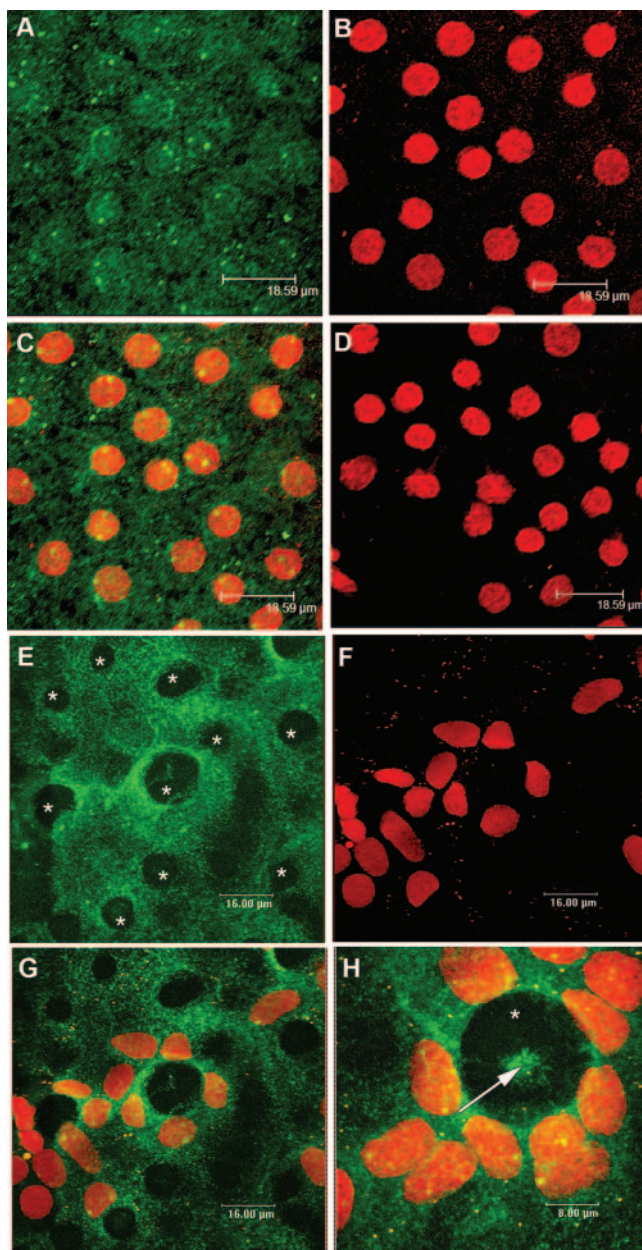


FIGURE 4. Representative confocal images of normal (A–D) and FED (E–H) endothelium in whole mounts of corneal tissue. (A) Uniform, punctate clusterin (CLU) staining (green) was present in normal corneal endothelium. (B) PI staining of nuclei (red) in the same tissue; (C) an overlay of the two images. (D) Negative control, incubated in secondary antibody only, showed no discernible staining for CLU. (E) CLU staining in FED-affected cells formed a rosette pattern around the dark areas, which represent corneal guttae (*). (F) PI-stained nuclei in the same tissue; (G) overlay of the two images. (H) Magnified view of FED endothelium 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: (A–G) 400 \times with 4 zoom; (H) 400 \times with 8 zoom.

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- to 40-kDa molecular mass range indicated that there were marked differences in the posttranslational modification of sCLU in FED versus normal samples.

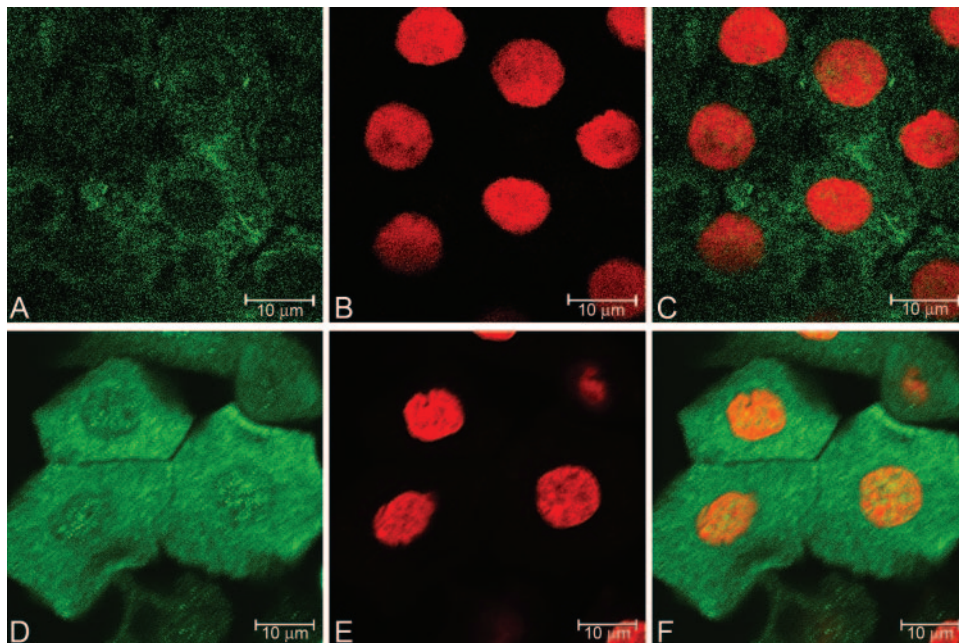
Western blot analysis further investigated CLU protein expression in both normal and FED endothelium. Expression of CLU in FED HCECs was significantly higher than in normal HCECs for both the nuclear and presecretory forms. Of interest was the finding that the level of the 30- to 40-kDa sCLU, which is 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 before extracellular secretion. Stressing the cells with proapoptotic 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 posttranslational 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 antiapoptotic effects of CLU by interfering with Bax activation in mitochondria.³⁹

FED-affected endothelial cells were also found to have elevated levels of nCLU compared with the normal cells. The nuclear CLU 49-kDa band was consistently present in FED, but not in normal cells by Western blot analysis. 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 pathologic 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 overexpression 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 overexpression 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 that amplifies all four CLU exons and detects the full length form of CLU.²⁷ There was a twofold increase in CLU cDNA in FED cells versus normal cells, indicating that there was an overall increase in CLU mRNA, as well as protein, expression in the diseased cells. The RT-PCR and Western blot data were 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 amplify specifically the nuclear form of CLU,²⁷ but could not obtain consistent results—a finding similar to that of other investigators (Michel D, Université de Rennes, Rennes, France, personal communication, 2007).³⁸ The mRNA analysis of the tissue taken from the pseudophakic bullous keratopathy specimens revealed a relative decrease in CLU production compared with 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 intracellu-

FIGURE 5. Representative confocal images taken through a single, 0.2- μm -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, C) and PI-staining of nuclei (red: B, E). Comparison of overlaid 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: $\times 400$ with 8 zoom.



lar 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 overexpressed in FED appears to be important in elucidating its pathophysiology. Overexpression 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 overexpressed in many pathologic conditions, two of which are Alzheimer's disease (AD) and 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 pathologic states point to a potentially common downstream pathway in these processes. Most studies 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 proapoptotic 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 increase with low, nontoxic, growth stimulatory levels of IR, and nCLU levels increase with much higher levels of the cytotoxic stress.¹⁵ Similar to the IR-induced CLU overexpression, both presecretory 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 may 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 indirectly points to the mechanism of FED pathogenesis involving oxidative stress-induced damage to the corneal endothelium.

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