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Co-localization of Increased Transforming Growth Factor Beta Induced Protein (TGFB β) and Clusterin Expression in Guttae of Fuchs Endothelial Corneal Dystrophy Patients

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Abstract

Purpose—To investigate the differential expression of TGFB β in normal human and Fuchs endothelial corneal dystrophy (FECD) endothelial cell-Descemet's membrane (HCEC-DM) complex, and to assess the structural role of TGFB β and clusterin (CLU) in guttae formation.

Methods—HCEC-DM complex was dissected from stroma in normal and FECD samples. Proteins were separated by 2-D gel electrophoresis and subjected to proteomic analysis. N-terminal processing of TGFB β was detected by Western blotting utilizing two separate antibodies against the N- and C-terminal regions of TGFB β . Expression of *TGFB β* mRNA was compared using real-time PCR. Subcellular localization of TGFB β and CLU in corneal guttae was assessed by fluorescence confocal microscopy.

Results—A major 68-kDa fragment and a minor 39-kDa fragment of TGFB β were identified on 2-D gels. Western blotting revealed an age-dependent proteolytic processing of the TGFB β N-terminus resulting in the increased formation of 57-kDa ($p=0.04$) and 39-kDa ($p=0.03$) fragments in older donors. FECD HCEC-DM showed a significant increase in the 68-kDa ($p=0.04$), 57-kDa ($p=0.01$), and 39-kDa ($p=0.03$) fragments of TGFB β . Real-time PCR analysis revealed that *TGFB β* mRNA was significantly increased ($p=0.04$) in FECD samples. TGFB β formed aggregates at the lower portions of guttae, next to the Descemet's membrane, while CLU localized mostly on top of the TGFB β -stained areas at the level of the endothelial cell nuclear plane.

Conclusion—The overexpression of pro-aggregative protein CLU, and pro-adhesive protein TGFB β , have been co-localized in the guttae. Such findings provide us with a better understanding of the major contributors involved in the aberrant cell-extracellular matrix interactions seen in the guttae of FECD patients.

Keywords

corneal endothelium; Fuchs endothelial corneal dystrophy; transforming growth factor beta induced protein (TGFB β); clusterin; proteomics; guttae

Fuchs endothelial corneal dystrophy (FECD) is the most common endogenous corneal endotheliopathy, leading to the progressive corneal edema, blindness and eventual need for corneal transplantation to restore vision. In early stages FECD is characterized by extracellular

collagenous deposits that accumulate posterior to Descemet's membrane, thicken it and cause the formation of mound-shaped aggregates, called "guttae." The characteristic pattern on specular microscopy showing dark areas in the endothelial mosaic is due to the displacement of the endothelium posterior to the plane of focus by these excrescences. In the later stages, the coalescence of the guttae occurs, and a progressive disruption of the endothelial mosaic causes cell thinning, stretching, enlargement, and loss of hexagonal shape.¹ Eventually, there is a compromise in the intact covering of the endothelial monolayer as endothelial cell loss progresses in an inversely proportional manner to guttae formation. The considerable strain on corneal endothelial cells situated at the apices of these excrescences causes the morphologic changes that lead to the loss of barrier function as well as cell apoptosis which has been widely implicated in FECD.² Still, the exact composition of these outgrowths or guttae is currently not clear.

Studies from this laboratory have described the results of 2-D gel analysis of proteins extracted from human corneal endothelial cell-Descemet's membrane (HCEC-DM) complexes dissected from the corneas of normal donors and FECD patients.^{3, 4} These studies showed that there is a marked overexpression of the pro-aggregative, chaperone-like protein, clusterin, in FECD.³ The staining of FECD corneas with CLU antibodies revealed a clustering of endothelial cells around guttae as well as CLU staining in the centers of guttae, suggesting the presence of cell remnants in these areas. Studies have shown that under stressed conditions CLU tends to cause cell aggregation and induce the formation of junctional contacts between cells.⁵ Results of the 2-D gel analysis also demonstrated a marked overexpression of a cell adhesion molecule, TGFBIp, in the FECD affected HCEC-DM complexes. TGFBIp was detected as a series of spots, migrating at ~38 kDa. The number and intensity of these spots was greater in FECD HCEC-DM as opposed to normal controls. The purpose of the current study is to further investigate the role of TGFBIp in the characteristic guttae formation and its relation to CLU.

TGFBI (transforming growth factor- β [TGF- β]-induced) gene encodes the transforming growth factor beta induced protein (TGFBIp), an extracellular matrix protein that mediates cell adhesion by interacting with collagens, fibronectin, and integrins, mainly $\alpha 3\beta 1$.⁶⁻¹⁰ This protein has been identified by *TGFBI* gene induction with TGF- β in a human adenocarcinoma line.¹¹ Mutations in the *TGFBI* gene are responsible for several corneal stromal dystrophies, such as Lattice dystrophy type I, Reis-Bucklers dystrophy, Thiel-Behnke corneal dystrophy, granular corneal dystrophy II (Avellino corneal dystrophy), and granular corneal dystrophy type I.¹²⁻¹⁵ Depending on the dystrophy, the mutation in the *TGFBI* gene manifest as stromal amyloid or/and nonamyloid deposits in which *TGFBI* itself has been co-localized.¹⁶ Analysis of the pathological corneas taken from the stromal dystrophy patients has revealed that there are mutation-specific changes in the processing of the full-size protein as opposed to unaffected corneas.¹⁶ In the current study, we employed immunocytochemistry to localize the TGFBIp deposition and to elucidate further the role of TGFBIp in guttae formation. Also, using antibodies against two non-overlapping parts of the protein we investigated whether there are changes in the processing of TGFBIp between FECD and normal endothelial samples. To obtain greater insight into the role of TGFBIp in the HCEC-DM complex we also compared the TGFBIp expression patterns in the corneas of young and old normal donors.

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 corneal dystrophy. After surgical removal of the FECD corneal buttons, two-thirds of the button was

used for the study and one-third was used for histopathological confirmation of the diagnosis. Normal human corneal buttons were obtained from the Tissue Banks International (Baltimore, MD) and National Disease Research Interchange (Philadelphia, PA) and were used as normal controls. The corneas were kept in storage medium for less than 72 hours before processing. We utilized our previously published criteria for obtaining normal controls from the tissue banks.^{3, 4}

Sample Preparation

Table 1 presents information regarding the normal and FECD tissue samples. Normal donors were decade-matched with FECD. Due to low protein or RNA content, two FECD samples contained 2 pooled corneas (Table 1, Sample 8 and 15). Corneal buttons were recovered from Optisol-GS 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 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

For 2-D gel electrophoresis the sample was prepared by pooling HCEC-DM protein extracts from 5 normal corneas of 3 donors (67 year-old female, 64 year-old male and 59 year-old male) (Table 1, Sample 1). Protein extract (150 µl) was loaded onto immobilized, pH 3-10 nonlinear gradient, 17 cm IPG strips (Bio-Rad, Hercules, CA, USA) for passive rehydration for 14 hrs. Isoelectric focusing and second dimensional separation were carried out as previously reported.^{3, 4} 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. Gel plugs from the protein 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. The identification of protein spots was performed by MALDI-TOF as previously described.^{3, 4} 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

Age difference in TGFBIp expression by Western blotting was compared between 3 young (2, 15, 18 year-old) and 3 older (64, 68, 73 year-old) normal donors (Table 1, Samples 2–7). TGFBIp expression was compared between 5 normal and FECD HCEC-DM samples (Table 1, Samples 8–12). Equal amounts of protein 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-TGFBIp (H-58) (Santa Cruz Biotechnology, Santa

Cruz, CA) diluted 1:100, goat polyclonal anti-TGFBIP (E-19) (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:100, and mouse monoclonal anti- β -actin (Sigma Aldrich, St. Louis, MO) diluted 1:6000 in blocking solution. The anti-TGFBIP antibodies were created against a recombinant TGFBIP (NCBI GI# 2498193), and molecular weight ranges were confirmed by the affinity purification of the specific peptides by Santa Cruz Laboratories, Santa Cruz, CA, 2008. Blots were rinsed, re-blocked, and exposed for 1 hour to horseradish peroxidase (HRP)-conjugated donkey anti-mouse IgG for β -actin, anti-rabbit IgG for clusterin, anti-rabbit IgG for TGFBIP (H-58), anti-goat IgG for TGFBIP (E-19). 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/ni-image>). Protein was normalized according to β -actin and total protein 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.

Real-Time PCR

Total RNA was extracted from normal and FECD HCEC-DM complexes (Samples 13–15, Table 1) as recommended by the manufacturer (TRIzol; Invitrogen). Samples were purified from DNA contamination by treating them with DNase I (Deoxyribonuclease I, Amplification Grade, Invitrogen). RNA quantity and quality were assessed by spectrophotometric analysis. For all samples, cDNA was prepared by reverse transcription using a commercially available kit (Promega). Relative expression levels of *TGFBIP* were assessed by real-time PCR using an ABI Prism 7900 HT sequence detection system instrument (Applied Biosystems, Foster, CA). Primers and probes for *TGFBIP* (TaqMan Gene Expression Assays, inventoried) and for the endogenous control β_2 -microglobulin (β_2 -MG) (human B2M endogenous control, FAM/MGB probe, TaqMan Endogenous Controls) were obtained from Applied Biosystems. Samples ($n=3$) were assayed in duplicate in a total volume of 50 μ L, using thermal cycling conditions of 2 minutes at 50°C and 10 minutes at 95°C followed by 50 cycles of 95°C for 15 seconds and 60°C for 1 minute. No template controls were run in each assay to confirm lack of contamination in reagents used for amplification. For data analysis, the comparative threshold cycle (C_T) method was adopted with the relative mRNA levels in normal subjects selected as the calibrator. The C_T was set in the exponential phase of the amplification plot. To normalize the amount of target gene in each sample, the difference in C_T (ΔC_T) was calculated by subtracting the average C_T of the endogenous control from that of the target gene. The amount of *TGFBIP* mRNA in FECD was expressed relative to the amount present in the calibrator, using the formula $2^{-\Delta\Delta C_T}$. Results were averaged and the standard error of the mean was 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.

Immunocytochemical Localization of TGFBIP and Clusterin

Normal and FECD corneas (Table 1, Samples 16–17) 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 in PBS, then permeabilized for 10 minutes with 1% Triton X-100 in PBS. Nonspecific binding was blocked using 4% bovine serum albumin (BSA; Fisher, Pittsburg, PA) in PBS for 10 minutes. Corneas were incubated for 2 hours in rabbit polyclonal anti-clusterin (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:50 in 4% BSA in PBS and goat polyclonal anti-TGFBIP (Santa Cruz Biotechnology, Santa Cruz, CA) 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-goat IgG (Jackson

ImmunoResearch) diluted 1:200 in 4% BSA in PBS, rhodamine-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch). Nuclear stain was performed by using TO-PRO-3 iodide (Molecular Probes, Eugene, Oregon) diluted 1:2000 in PBS. Negative controls consisted of secondary antibody alone. After being washed in PBS, corneas were placed endothelial-side up on slides using mounting medium (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 μm 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

Analysis of TGFBIp Isoform Expression in Normal HCEC-DM Complex

Our previous studies comparing the proteome of FECD and normal corneal endothelium revealed a marked overexpression of TGFBIp in 2D gels of FECD samples. In this study, we further investigated TGFBIp expression and proteolytic processing in normal human corneal endothelium. To characterize TGFBIp expression, HCEC-DM protein extracts from three normal donors were pooled and then subjected to 2D-gel electrophoresis followed by MALDI-TOF analysis. MALDI-TOF identified TGFBIp in two areas of the gel. TGFBIp was identified as a series of spots with different pI migrating at two different relative molecular weights. The first row of spots identified as TGFBIp was located at the 68-kDa range within pI 3.0-8.0 as shown in brackets in Figure 1A. The major 68-kDa TGFBIp fraction constituted a series of spots that most likely represent different posttranslational modifications of TGFBIp. The second series of spots identified as TGFBIp was at 39 kDa range as shown in the lower brackets of Figure 1A. The 39-kDa fragment was formed by 6 spots within pI 4.5-6.0, apparently representing different posttranslational modification of this TGFBIp form as well. The analysis of the remainder of the spots on the 2 D-gel did not reveal any additional TGFBIp forms.

TGFBIp processing was studied by Western blotting with antibodies reactive to two non-overlapping parts of the TGFBIp. Figure 1B depicts the diagram of anti-TGFBIp 120-170 (E-19) and anti-TGFBIp 626-683 (H-58) binding sites (molecular weight range as provided by direct communication with Santa Cruz, Santa Cruz, CA, 2008). Immunostaining of one-dimensional gels with anti-TGFBIp 120-170 and anti-TGFBIp 626-683 antibodies revealed a major band migrating at 68 kDa, which corresponded to the full-size protein seen on the 2-D gel (Figure 1C and D). The anti-TGFBIp 626-683 which reacts with the C-terminus of TGFBIp revealed several minor species of TGFBIp. The molecular weights of the TGFBIp fragments found on the blots were very similar to the previously published molecular weights of TGFBIp fragments found in the protein extracts of the whole corneas.¹⁶ Apart from the 68-kDa protein, anti-TGFBIp 626-683 recognized fragments of 57-, 39-, and 29-kDa. The 39-kDa fragment corresponded to relative molecular weight of the minor TGFBIp species seen on the 2-D gel. In contrast, anti-TGFBIp 120-170 which specifically reacts with the N-terminus of TGFBIp, did not detect these minor TGFBIp species. This differential staining suggests that minor TGFBIp species not detected with the antibody against the N-terminus represent N-terminal deletions during the processing of the full-size protein.

Analysis of Age-related Differences

To explore the age-related differences in TGFBIp expression, six normal corneas at different ages were analyzed with the Western blotting (Table 1, Sample 2–7). The immunostaining with anti-TGFBIp 626-683 antibody to the C-terminus revealed a different staining pattern at different donor ages. Figure 2A shows a representative blot of 2, 15, 64, and 73 year-old donors, 2B presents the densitometric comparison of all 6 samples based on normalization to β -actin, and 2C presents the densitometric comparison of all 6 samples based on normalization to total

protein amount. The comparison of protein levels between young and old donors revealed similar results when TGFBIp content was normalized to β -actin or to total protein amount. The major band at 68-kDa range was present in all ages. The 57-kDa band was present only in older donors ($p=0.04$), indicating that this fragment of TGFBIp is formed due to age-related proteolytic processing. On average, there was a 2-fold increase in the 39-kDa band in older individuals ($p=0.03$). The 29-kDa band was also present only in older donors but it showed variable intensity between the specimens rendering it not statistically significantly elevated in older HCEC-DM complexes ($p=0.05$) when TGFBIp content was normalized to β -actin, but statistically significantly elevated in older samples ($p=0.01$) when TGFBIp content was normalized to total protein content.

Differential expression of TGFBIp forms in Normal and FECD Endothelium

Prior proteomic studies revealed a significant overexpression of TGFBIp in FECD samples on 2D- gels.³ To investigate further the differential expression of TGFBIp forms in FECD and normal HCEC-DM samples, Western blot analysis was performed with antibodies against N- and C-termini of TGFBIp. The Western blot data obtained from 1 pair of pooled samples (Table 1, Sample 8) and 4 pairs of non-pooled samples (Table 1, Samples 9–12) was identical. Figure 3 shows a representative blot with anti-TGFBIp for the C-terminus (3A) and a representative blot with anti-TGFBIp for N-terminus (3B), and Figure 3C presents the densitometric comparison. There was a marked overexpression of most TGFBIp forms in FECD specimens as opposed to normals. On average, there was a 5-fold increase in the full-size protein (68-kDa band) in FECD ($p=0.04$), as well as statistically significant increase in minor TGFBIp species: 57-kDa band ($p=0.01$) and 39-kDa band ($p=0.03$) seen in FECD. The 29-kDa TGFBIp band was increased in FECD endothelium but not at a statistically significant level ($p=0.11$). In order to compare protein levels between FECD and normal specimens, results were normalized to β -actin. The 3rd column in the blots shows a normal specimen loaded at a twice as high protein concentration as in the first two columns (Figure 3A–B). Such higher loading was performed to illustrate that both FECD and normal samples generally exhibited the same TGFBIp species but at higher levels in FECD when loading was normalized with β -actin. The 29-kDa band had variable and faint presence in most normal samples. The single band (68-kDa) that stained with anti-TGFBIp 120-170 antibody was also markedly increased in FECD, corroborating the fact the full-size TGFBIp is overexpressed in FECD. No other bands were detected with this antibody to the N-terminus. FECD specimens demonstrated an increase in levels of all these fragments but did not reveal any new or unusual forms of TGFBIp.

Real-time PCR Comparing *TGFBI* Expression between Normal and FECD HCEC-DM

Proteomic analysis and Western blot data revealed the up-regulation of TGFBIp expression in FECD-affected corneal endothelium. To investigate this difference, real-time PCR was performed to evaluate the mRNA level of *TGFBI*. The PCR analysis was performed by using previously optimized primers and probes from Applied Biosystems. Three different samples (Samples 13–15) were used to compare the *TGFBI* mRNA expression between FECD and normal controls. The real-time PCR showed an up-regulation of *TGFBI* mRNA levels in FECD samples when normalized with the internal control, β 2-MG (Figure 4). The mean $\pm$ SEM relative expression of *TGFBI* mRNA in FECD group (2.35 ± 0.23) was significantly higher with that in normal subjects (1.31 ± 0.38) ($P=0.04$).

Co-localization of TGFBIp and Clusterin in FECD

Previous immunofluorescence studies showed a characteristic CLU staining pattern in FECD endothelium with a rosette-type clustering of endothelial cells around dark centers representing corneal guttae. Indirect immunofluorescence analysis was performed to investigate the relationship between TGFBIp and CLU and to compare their localization in the endothelium

of normal and FECD donors. Figure 5 presents confocal images in which the Z-series was collapsed onto a single image plane. Figure 5A – D presents confocal images of normal endothelium. In normal tissue, a relatively uniform, punctate distribution of TGFBIp and CLU was observed within the cytoplasm (Figure A, B). Descemet's membrane revealed bright fluorescence with anti-TGFBIp antibody in all the specimens (data not shown). Interestingly, the TGFBIp staining pattern in FECD endothelium (Figure 5E–H) was different from that of normal HCEC. The dark areas which did not contain the nuclei and were considered to be corneal guttae had significant staining with both TGFBIp and CLU in FECD samples (Figure 5EH, asterisks). In the centers of guttae, TGFBIp co-localized with CLU most of the time (Figure 5E–H, arrows). FECD tissue showed a much lower cell density than normal tissue; diffuse but less bright TGFBIp staining was visible even outside defined guttae indicating the staining of the Descemet's membrane in areas devoid of cells. Negative controls consisted of normal corneas incubated with secondary antibody only and no TGFBIp or CLU-positive staining of either cells or Descemet's membrane was observed under these conditions, indicating the specificity of primary antibody staining (Figure 5I–J).

To explore further the relationship between CLU and TGFBIp staining single Z-plane images were taken through the guttae of FECD tissue at various depths (Figure 6). A representative image through the apical part of endothelium at the level of the nuclear plane showed intense intracellular, intranuclear and extracellular staining with CLU (Figure 6B, D). The extracellular staining was most abundant in the center of the depicted gutta. At this particular plane, there was only faint staining in the center of the gutta with anti-TGFBIp antibody (Figure 6A). Figure 6E–H represents the images that were taken 1.0 μm deeper into the cornea. At this plane, both TGFBIp and CLU staining co-localized in the center of the gutta. Figure 6I–L represents the images that were taken 3.8 μm deeper into the cornea beyond the nuclear plane of the same gutta. At this plane, the center of the gutta had bright staining with anti-TGFBIp antibody. The images taken at an even deeper level showed significant TGFBIp staining at the level of Descemet's membrane (data not shown). Overall, the images manifested specific co-localization of TGFBIp with CLU in the centers of corneal guttae of FECD specimens. TGFBIp tended to localize to the lower portions of guttae next to the Descemet's membrane and CLU localized on top of the TGFBIp-stained areas.

Discussion

The transforming growth factor beta induced protein is mainly an extracellular matrix protein that has a secretory signal sequence at the N-terminus, four homologous internal domains and a cell attachment site (consisting of RGD amino acids) at the C-terminus.⁶ The main function of TGFBIp is attributed to its ability to bind various ligands and mediate cell adhesion via the RGD sequence.⁷ Our previous studies detected a marked overexpression of clusterin and TGFBIp in FECD endothelium.³ In the current study, we performed a targeted analysis of TGFBIp processing in normal and FECD HCEC-DM and compared TGFBIp expression in young and old donors. The co-localization of TGFBIp and CLU expression in guttae provided us with an insight into the structural composition of these extracellular excrescences.

Proteomic analysis of normal HCEC-DM showed that TGFBIp is abundantly expressed in the endothelial-DM complex. TGFBIp has multiple threonine, tyrosine and serine sites which allow phosphorylation and thus posttranslational modification.¹⁶ On 2-D gels, 68- and 39-kDa TGFBIp fragments migrated as series of spots with different pI's most likely representing different posttranslational modifications of TGFBIp. This finding is in agreement with the other studies that performed 2-D electrophoresis on the whole corneal extracts and identified TGFBIp in multiple spots migrating at the same molecular weight, but with different pI's.¹⁶ The fact that the 57- and 29-kDa fragments were repeatedly identified by Western blotting but

not on the 2-D gels is most likely due to these forms being present in much lower concentrations and to MALDI-TOF not being sensitive enough to identify them.

The analysis of TGFBIp content using antibodies against two non-overlapping parts of the protein revealed the presence of C-terminal fragments of the protein that varied in relative concentration with increasing age of the donors. In addition to the major 68-kDa band corresponding to the full-size protein, we identified a set of fragments that are most likely the proteolysis products characteristic for TGFBIp turnover in normal HCEC-DM. It is possible that the TGFBIp fragments were created from degradation by extracellular proteases. Nevertheless, a more plausible explanation is the fragment formation from normal TGFBIp turnover in corneal endothelium, since almost identical molecular weight fragments were detected in the study by Korvatska et al., in which N-terminal sequencing and immunostaining were performed to characterize TGFBIp content in the whole corneas.¹⁶ Our study showed an increase in the levels of the 39-kDa fragment with increasing age of donors. Of note, only 68- and 39-kDa bands were present throughout the age range of donors. The other fragments (57-kDa and 29-kDa) appeared to be present in older age samples only, implicating the age-related differences in normal TGFBIp turnover. The gradual thickening of Descemet's membrane and building of the posterior nonbanded layer with age is in agreement with increasing accumulation of TGFBIp which is known to have a physiological interaction with collagens.^{7, 17, 18} Previous studies performed on whole rabbit corneal buttons showed an increase in steady state levels of *TGFBI* mRNA that correlated with increasing rate of collagen accumulation during corneal morphogenesis.¹⁸ Several immunohistochemical studies have localized TGFBIp in normal Descemet's membrane, posterior collagenous layer, and retrocorneal fibrous membrane inferring its role as a structural element of the aging and injured Descemet's membrane.^{19–22} The exact role of TGFBIp is not known but its abundance in the extracellular milieu of endothelium implies a substantial role in the cell-Descemet's membrane interaction.

Previous study comparing 2-D gels of FECD and normal donors revealed an increase in TGFBIp spot intensity and number in FECD samples.³ To analyze this difference further we performed Western blot analysis comparing the TGFBIp expression in normal and FECD HCEC-DM. Expression of 68-, 57-, and 39-kDa fragments was statistically significantly higher in the FECD HCEC-DM. The expression of the 29-kDa fragment was elevated in FECD but not at a significant level. To corroborate the increase of TGFBIp levels in FECD, its expression at the gene level was compared between normal and FECD samples. The finding that levels of *TGFBI* mRNA were significantly increased in FECD samples further substantiates the proteomic data and indicates that the source of the differences stem from increased gene transcription.

Studies analyzing pathologic stromal deposits due to mutation in *TGFBI* gene have also identified an accumulation of TGFBIp fragments in dystrophic corneas as compared to normal controls.^{16, 23, 24} TGFBIp has been shown to co-localize and co-aggregate into these deposits which were composed of either amyloid or non-amyloid depending on the form of stromal dystrophy.¹⁶ In addition to the increase in TGFBIp production, the analysis of these corneas detected overexpression of aberrant forms of TGFBIp that varied in their molecular weight depending on the type of dystrophy. In our study of FECD samples, we did not detect any aberrant or unusual forms of TGFBIp that were not present in normal age-matched controls, implying that probably there is no intrinsic mutation in the *TGFBI* gene causing the accumulation of the protein. This is in contrast to the findings in the stromal dystrophies where overexpression of both normal and aberrant forms of TGFBIp has been attributed to the *TGFBI* gene mutations.¹⁶

The immunohistological analysis revealed an interesting pattern of CLU and TGFBIp colocalization in the guttae, the excrescences characteristic of Fuchs corneal dystrophy. The staining for TGFBIp was prominent throughout the Descemet's membrane and showed a marked increase in intensity at the centers of the guttae. CLU was also present in the centers of the guttae but on top of TGFBIp staining, closer to the apical side of the endothelium. The diagram in Figure 7 shows a schematic representation of the relationship of CLU and TGFBIp role in guttae formation. This is the first known study co-localizing CLU and TGFBIp in these pathologic extracellular matrix deposits. There is no known correlation in the literature between clusterin and TGFBIp. It is known though, that CLU overexpression at the times of cellular stress (including oxidative stress) causes the cells to aggregate via cell-cell and cell-substratum interactions.⁵ The driving force of these interactions often times is the production of cell adhesive molecules and junctional complexes. In a renal injury model, such interactions are capable of preserving the integrity of the renal epithelial barrier.^{25, 26} When the cell-matrix interactions are disrupted, a form of apoptosis called 'anoikis' ensues. Similarly in FECD, there is an over-production of the cell adhesion molecule, TGFBIp in the setting of the prolonged tissue injury and pathological CLU overexpression. It is possible, that the endothelial cells under stressors of the dystrophic degeneration are 'clumping' with one another via the action of CLU and are adhering to their substratum via the action of TGFBIp. The attempt of the cells to attach to their substratum is progressively disrupted during guttae formation, thus rendering the cells susceptible to apoptosis.

In conclusion, there is an increased production and modification of TGFBIp in the aging HCEC-DM complex. The increase in TGFBIp production is even greater in FECD as compared to the age-matched normal controls. The pro-aggregative protein CLU, and pro-adhesive protein TGFBIp, co-localize in the centers of guttae. Even though such findings cannot infer the functional role of these proteins in the pathogenesis of the dystrophy, it provides us with the better understanding of the major culprits involved in the aberrant cell-extracellular matrix interactions.

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Figure 1. TGFB1p expression in normal human HCEC-DM

SYPRO Ruby staining of a two-dimensional gel of HCEC-DM proteins pooled from 5 corneas of 3 normal donors (A). The group of TGFB1p spots at 68 kDa and 39 kDa range are indicated within brackets. The position of β -actin is indicated by the arrow. The diagram of anti-TGFB1p 120-170 and 626-683 antibody binding is presented in a diagram (B). Western blotting of HCEC-DM protein extracts resolved on one-dimensional gels with anti-TGFB1p 626-683 antibody (C), and anti-TGFB1p 120-170 antibody (D). The long arrows represent the alignment of 68-kDa and 39-kDa TGFB1p fragments resolved on a 2-D gel and a Western blot.

Figure 2. Age-related TGFBIp expression pattern

Representative Western blots comparing anti-TGFBIp 626-683 expression of HCEC-DM extracts from normal 2, 15, 64, and 73 year-old (yo) donors (A). β -actin was used for normalization of protein loading. (B) Densitometric comparison of the average expression of TGFBIp fragments (Table 1, Sample 2–7). Bars: SD.

Figure 3. Comparison of TGFBIp expression patterns between FECD and normal HCEC-DM Representative Western blots comparing with anti-TGFBIp 626-683 (A) and anti-TGFBIp 120-170 (B) comparing TGFBIp expression in FECD and normal HCEC-DM (Table 1, Sample 12). The same normal sample was used in the second and third columns but twice as high loading in the 3rd column. β -actin was used for normalization of protein loading. (C) Densitometric comparison of the average expression of TGFBIp fragments based on the anti-TGFBIp 626-683 results. Bars: SD. *P=0.04; **P=0.01; ***P=0.03; n.s.=not statistically significant.

Relative expression of KE mRNA

Figure 4. Comparison of *TGFBI* gene expression between FECD and normal HCEC-DM by real-time PCR

Mean relative expression of mRNA in normal subjects and in patients with FECD. Bars: SEM.

*P=0.04.

Figure 5. Co-localization of TGFBIp and CLU in normal and FECD corneal endothelium

Representative confocal images of normal (A–D) and FECD (E–H) endothelium in whole mounts of corneal tissue. Uniform TGFBIp staining (green) and CLU staining (red) was present in the cytoplasm of normal corneal endothelium (A–B). (C) TO-PRO-3 staining of nuclei (blue); (D) an overlay of the three images. (E–H) The guttae which did not contain the nuclear stain were denoted with asterisks. (E) TGFBIp staining (arrows) in FECD-affected cells with increased brightness in the areas of guttae (asterisks). (F) CLU staining (arrows) in the same tissue which co-localized to the TGFBIp-stained areas in the centers of guttae (asterisks). (G) TO-PRO-3-stained nuclei in the same tissue with nuclei surrounding guttae (asterisks); (H) overlay of the three images co-localizing TGFBIp and CLU (arrows). Negative control, incubated in secondary antibodies, shows no discernable staining for TGFBIp and CLU in normal (I) and FECD (J) samples. Final magnification 400× with 3 zoom.

Figure 6. Localization of TGFBIp and CLU in the gutta

Representative confocal images taken through a single, 0.2 μm-thick z-plane of gutta at the level of the endothelial nuclear plane (A–D), 1.0 μm (E–H), and 3.8 μm (I–L) deeper towards the Descemet’s membrane. Images were taken after immunostaining for TGFBIp (*green*) (A, E, and I); CLU (*red*) (B, F, and J); TOPRO-3 (*blue*) (C, G, and K); the overlay of the three images (D, H, and L). Final magnification 400× with 8 zoom.

Figure 7. Schematic representation of the role TGFBIp and CLU in guttae formation

There is a gradually increasing production of TGFBIp and CLU in the centers of guttae. TGFBIp accumulates in close proximity to the Descemet's membrane, while CLU forms aggregates on top of the TGFBIp deposits at the endothelial cell level. Both TGFBIp and CLU co-localize in the middle of the guttae (circular insert).

Table 1

Donor Information

Sample #	FED HCEC-DM†		Normal HCEC-DM		Use of Samples
	Age	Sex	Age	Sex	
1			67	F	2-D gel
			64	M	
			59	M	
2			2	M	Western blot analysis
3			15	F	
4			18	M	
5			64	F	
6			72	F	
7			73	M	Western blot analysis
8	69	M	67	F	
	66	F	72	F	
9	75	F	73	M	
10	55	F	68	M	
11	78	F	73	M	Real-time PCR
12	79	F	72	F	
13	69	F	68	F	
14	59	M	69	M	
15	72	M	64	F	
	69	F			Immunohistochemistry
16	64	F	65	F	
17	87	F	71	M	

FED: Fuchs' endothelial dystrophy
HCEC-DM: Human corneal endothelium and Descemet's membrane