Increased Endoplasmic Reticulum Stress in Human Glaucomatous Trabecular Meshwork Cells and Tissues

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Submitted: December 8, 2014
Accepted: May 6, 2015

Citation: Peters JC, Bhattacharya S, Clark AF, Zode GS. Increased endoplasmic reticulum stress in human glaucomatous trabecular meshwork cells and tissues. *Invest Ophthalmol Vis Sci*. 2015;56:3860–3868. DOI:10.1167/iovs.14-16220

PURPOSE. Primary open-angle glaucoma (POAG) is the most common form of glaucoma and is accompanied by elevated intraocular pressure (IOP) resulting from increased aqueous humor outflow resistance through the trabecular meshwork (TM). The pathological mechanisms underlying increased outflow resistance have not been fully delineated. We recently demonstrated that chronic endoplasmic reticulum (ER) stress in the TM is associated with ocular hypertension in mouse models of glaucoma. The purpose of this study was to determine whether ER stress is also increased in human glaucomatous TM cells and tissues.

METHODS. Endoplasmic reticulum stress markers including GRP78, GRP94, and C/EBP homologous protein (CHOP) were examined by immunohistochemistry in the TM of age-matched normal ($n = 18$) and open-angle glaucoma donors ($n = 18$). GRP78, GRP94, activating transcription factor (ATF)-4, endoplasmic oxidoreductin-1 α (ERO-1 α), phosphorylated eukaryotic translation initiation factor 2 α (EIF-2 α), and CHOP were examined by Western blot analysis in TM tissue lysates from age-matched normal ($n = 4$) and POAG donors ($n = 5$). In addition, ER stress markers were examined in primary TM cells isolated from normal ($n = 4$ NTM) and glaucoma ($n = 4$ GTM) human donors.

RESULTS. Immunohistochemical analysis demonstrated a significant increase in GRP78 and GRP94 in the glaucomatous TM ($n = 18$) compared to normal TM ($P < 0.0001$, $n = 18$). Interestingly, there was minimum CHOP immunostaining observed in normal TM tissues. However, there was a 3-fold increase in CHOP levels in the glaucomatous TM ($P < 0.0001$; $n = 18$), indicating the presence of chronic ER stress in the glaucomatous TM. Western blot analysis of TM tissue lysates also demonstrated increased ER stress markers in the glaucomatous TM tissues including GRP78, GRP94, ATF-4, ERO-1 α , and CHOP. Densitometric analysis of Western blots showed a significant increase in ATF-4, ERO-1 α , and CHOP expression in the glaucomatous TM ($n = 5$) compared to age-matched normal TM ($n = 4$). In addition, primary TM cells obtained from glaucoma donors demonstrated increased ER stress markers including increased GRP78, GRP94, ATF-4, ERO-1 α , and CHOP compared to normal TM cells. However, glaucomatous TM cells did not show splicing of XBP-1, a marker of unfolded protein response pathway.

CONCLUSIONS. These studies indicate the presence of chronic ER stress in human glaucomatous TM tissues and cells and further suggest that ER stress pathway may provide a novel target for developing disease-modifying glaucoma treatments.

Keywords: ER stress, trabecular meshwork, CHOP, glaucoma

Glaucoma is a group of optic neuropathies characterized by progressive loss of retinal ganglion cell (RGC) axons and irreversible loss of vision.^{1,2} It is the second leading cause of irreversible blindness in the world, affecting approximately 70 million people.^{3,4} Primary open-angle glaucoma (POAG) is the most common form of glaucoma, accounting for approximately 70% of all cases.¹ Elevated intraocular pressure (IOP) is a major associated risk factor and also a main target for treatment.^{5,6} Elevated IOP is caused by increased aqueous humor outflow resistance through the drainage structure of the trabecular meshwork (TM).^{7,8} The mechanisms that cause increased outflow resistance in the TM are not well understood.

The endoplasmic reticulum (ER) is involved in the synthesis and processing of secreted and membrane proteins. Various

physiological and pathological conditions can disturb protein folding in the ER, causing ER stress.⁹ To alleviate such cellular stress, eukaryotic cells activate a cytoprotective response known as the unfolded protein response (UPR) pathway.^{9,10} Activation of the UPR involves sensing of ER stress via inositol requiring protein 1 (IRE1), PKR-like endoplasmic reticulum kinase (PERK), and activating transcription factor (ATF)-6 α . Activation of PERK phosphorylates eukaryotic translation initiation factor 2 α (EIF-2 α), which is involved in protein translational attenuation and reducing ER protein load. Activation of PERK is also involved in induction of activating transcription factor 4 (ATF-4), which induces expression of genes involved in restoring ER homeostasis. Activated IRE1 converts XBP-1 pre-mRNA to mature mRNA via an unconven-

tional splicing mechanism. Processed XBP-1 acts as a transcriptional activator of many genes, including genes involved in protein folding, quality control, and regulation of ER/Golgi biogenesis.¹¹ Activation of ATF-6 α involves its translocation to Golgi apparatus for proteolytic cleavage to release active ATF-6 α , which controls the expression of UPR genes.¹⁰ When the UPR adaptive response is not sufficient to resolve defects in protein folding and chronic ER stress persists, ER dysfunction can lead to cell death. Excessive and sustained ER stress leads to activation of cell death/dysfunction via induction of C/EBP homologous protein (CHOP), ER-specific caspase 12, and several other factors.^{12,13} C/EBP homologous protein, a transcriptional factor, is a significant mediator of apoptosis in response to ER stress.¹⁴ Transcriptional induction through ATF-4 and CHOP enhances protein synthesis leading to oxidative stress and cell death.¹⁵ Increased CHOP also promotes oxidation of ER proteins via upregulation of endoplasmic oxidoreductin-1 α (ERO-1 α), which encodes for an ER oxidase.¹⁶ Increased oxidation in ER can favor protein misfolding and ER dysfunction, leading to cell death.

Endoplasmic reticulum stress has been shown to be involved in the pathophysiology of various diseases including diabetes, inflammation, kidney disease, liver disease, and neurodegenerative diseases.⁹ The role of ER stress in the pathophysiology of glaucoma has not been fully characterized. Recently, we linked ER stress to the pathogenesis of myocilin-associated glaucoma.¹⁷ Mutations in the myocilin gene (*MYOC*) are the most common known genetic cause of glaucoma.¹⁸ *MYOC* mutations resulting in elevated IOP are responsible for ~4% of POAG and most cases of autosomal dominant juvenile-onset open-angle glaucoma.¹ Recently, we developed a transgenic mouse model (*Tg-MYOC^{Y437H}*) by expressing human *MYOC* containing the Y437H mutation under the control of the cytomegalovirus (CMV) promoter. This mouse model expresses mutant myocilin in relevant eye tissues and displays relatively early-onset glaucoma phenotypes closely resembling those seen in POAG patients. Using *Tg-MYOC^{Y437H}* mice, we determined that ER stress plays a key role in the in vivo pathogenesis of *MYOC*-associated glaucoma. Expression of mutant myocilin induced ER stress and activated UPR in the TM in vitro and in vivo. However, the failure of the UPR to resolve this ER stress led to chronic and dysregulated ER stress, inducing death of TM cells associated with upregulation of the transcriptional factor, CHOP. Mitigation of ER stress by systemic or topical administration of the chemical chaperone sodium 4-phenylbutyric acid (PBA) rescued glaucoma phenotypes in *Tg-MYOC^{Y437H}* mice.^{17,19}

Our recent work also showed that ER stress contributes to the pathology of glaucoma not only in myocilin-associated glaucoma but also in another model of glaucoma. Specifically, we have shown that ER stress plays an important role in glucocorticoid-induced ocular hypertension.²⁰ Recently, we demonstrated that topical ocular dexamethasone (0.1%) treatment induced ocular hypertension and resulted in open-angle glaucoma in otherwise healthy C57BL/6 mice, similar to steroid glaucoma patients. Dexamethasone induced ER stress and activated UPR in TM cells in vitro and in vivo. Chronic dexamethasone treatment also induced CHOP, and deletion of CHOP prevented IOP elevation in this mouse model. Furthermore, reduction of ER stress by PBA reduced IOP elevated by dexamethasone.

Conditions that can trigger ER stress are aging,²¹ oxidative stress,²² expression of unfolded or misfolded proteins,^{23,24} and increased synthesis of secretory proteins.^{17,20,25–27} Several of these conditions are also associated with the pathogenesis of glaucoma. Unfolded protein response genes (*PDI*A5 and *BIRC6*) harbor risk alleles for POAG,²⁷ suggesting that association of these DNA variants may compromise the UPR

response in the TM. Either mutant myocilin or dexamethasone treatment increases ER chaperones including GRP78 and GRP94 in the TM. However, failure of the TM to eliminate ER stress results in induction of CHOP, which may be involved in TM cell death and IOP elevation. Therefore, we hypothesize that the unfolded protein response of the TM is insufficient to prevent abnormal protein accumulation over time resulting in chronic ER stress and induction of CHOP, causing dysfunction and eventual loss of TM cells, thus elevating IOP. In the present study, we sought to examine whether the above ER stress markers are increased in human glaucomatous TM tissues.

METHODS AND MATERIALS

Primary TM Cell Cultures

Human TM cells were obtained from Abbot Clark's laboratory (University of North Texas Health Science Center) and cultured as described previously.^{28,29} For experiments, TM cells were further grown in Dulbecco's modified Eagle's medium (DMEM) containing L-glutamine (0.292 mg/mL; Invitrogen-Gibco, Grand Island, NY, USA), penicillin (100 units/mL)/streptomycin (0.1 mg/mL; Invitrogen-Gibco), and 10% fetal bovine serum (Invitrogen-Gibco). For ER stress analysis, three normal TM cell strains and four glaucomatous TM cell strains (between passages 4 and 8) were grown in serum-free medium for 48 hours, and total cell lysates were collected and subjected to Western blot analysis as described previously.²⁰ For XBP-1 splicing, four normal and four glaucomatous TM cell strains were grown to confluence and total RNA was subjected to XBP-1 RT-PCR. For dexamethasone treatment, TM cells were incubated with fresh medium containing 100 nM dexamethasone (Sigma-Aldrich Corp., St. Louis, MO, USA) for 10 days. Trabecular meshwork cells were replenished with fresh media containing 100 nM dexamethasone every 2 days. Total RNA was isolated and subjected to RT-PCR of XBP1.

Analysis of ER Stress Markers in Human TM Tissues

Endoplasmic reticulum stress markers were examined by immunohistochemistry and Western blot for the selected ER stress markers. The KDEL antibody recognizes the SEKDEL amino acid sequence present in ER resident proteins including GRP78 and GRP94 with particular prominence. In Western blot analysis, KDEL antibody recognizes only GRP78 and GRP94 protein. Previously, we reported increased KDEL staining indicating increased GRP78 and GRP94 in the TM of *Tg-MYOC^{Y437H}* mice.¹⁷ Therefore, we utilized KDEL and CHOP immunostaining for analysis of ER stress in the human TM. Since several of the ER stress marker antibodies did not work well on paraffin-embedded sections, we examined GRP78, GRP94, pEIF-2 α , ATF-4, and CHOP by Western blot analysis of TM tissue lysate as demonstrated previously.²⁰ It should be noted that selected ER stress markers were chosen because these proteins are commonly used as ER stress markers and we have shown these markers increased in the TM of mouse models of glaucoma.^{17,20} Endoplasmic reticulum stress sensors including IRE, PERK, and ATF-6 are not used because detection of these proteins normally requires Western blot analysis on large amounts of total protein, which is very difficult to obtain from TM tissue lysates.

Immunohistochemistry

Age-matched normal ($n = 18$) and open-angle glaucoma ($n = 18$) donor eyes as listed in Table 1 were used. Although the

TABLE 1. List of Normal and Glaucoma Eyes Used for Immunostaining

No.	Race	Age	Ocular Disease
00-23	C	74	Normal; IOL ou
01-48	C	78	Normal, macular degeneration
04-103	C	79	Normal
241-10	C	77	Normal
03-155	C	89	Normal
04-116	C	82	Normal
183-11	C	80	Normal
1193-09	C	88	Normal, cataract
1210-09	C	84	Normal, cataract
04-112	C	82	Normal
371-11	C	85	Normal
446-11	C	84	Normal
839-10	C	84	Normal
842-09	C	82	Normal, intraocular lenses
1187-09	C	81	Normal
141-11	C	90	Normal
1192-09	C	96	Normal
05-358	C	92	Normal
05-125	C	83	Open-angle glaucoma
49-11	C	78	Open-angle glaucoma, diagnosed 1 y ago
114-11	C	70	Open-angle glaucoma; glaucoma implants, AMD, diabetic retinopathy
140-11	C	76	Open-angle glaucoma
04-218	C	86	Open-angle glaucoma
304-11	C	89	Open-angle glaucoma, bilateral cataract surgery
936-10	C	82	Open-angle glaucoma
1104-11	C	85	Open-angle glaucoma; diabetic retinopathy, partial blindness
1109-10	C	80	Open-angle glaucoma
04-128	C	83	Open-angle glaucoma
04-223	C	86	Open-angle glaucoma
05-342	C	87	Open-angle glaucoma
50-11	AA	83	Open-angle glaucoma
115-11	C	81	Open-angle glaucoma
413-11	C	89	Open-angle glaucoma, Mac. Deg.
04-05	C	93	Open-angle glaucoma
05-2	C	95	Open-angle glaucoma
138-10	C	91	Open-angle glaucoma, wet macular degeneration, bilateral cataract

C, Caucasian; AA, American Asian; IOL, intraocular lenses; Mac. Deg., macular degeneration; ou, both eyes; AMD, age-related macular degeneration. Note that most of the glaucoma donor eyes are presumably from POAG donors. However, due to lack of proper documentation, we referred them as open-angle glaucoma.

majority of the glaucomatous TM tissues were from POAG donors, due to lack of proper documentation we referred to them as open-angle glaucoma donors. Age groups (70–80 years, $n = 4$ each; 80–90 years, $n = 11$ each; 90–100 years, $n = 3$ each) were compared among groups as well as all together. Briefly, eyes were obtained from regional eye banks within 6 hours of death and fixed in 10% formalin. The eyes were obtained and managed in compliance with the Declaration of Helsinki. Tissues were dehydrated and embedded in paraffin and stored until further use. The slides were deparaffinized in xylene and dehydrated twice with 100%, 95%, 70%, 50% ethanol for 5 minutes. Slides were then incubated in 100°C citrate buffer (pH 6.0) for 13 minutes followed by room temperature citrate buffer (pH 6.0) for 13 minutes. Nonspecific staining of the tissues was blocked by incubation in SuperBlock blocking buffer (Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA) for 30 minutes in a dark, humid chamber. Sections were incubated overnight at 4°C with rabbit CHOP antibody (1:50 in blocking buffer, catalogue no. SC-575; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and mouse KDEL antibody (1:50 in blocking buffer, catalogue no. ab12223; Abcam, Inc., Cambridge, MA, USA). Sections were washed with PBS and incubated for 2 hours at room temperature with Alexa donkey anti-rabbit 488 and Alexa donkey anti-mouse

568 (1:500 in PBS; Thermo Fisher Scientific, Inc.). Sections were washed with PBS and mounted with mounting media containing diaminophenylindole (DAPI; Vector Labs, Inc., Burlingame, CA, USA). Z-stacks were acquired in a confocal microscope (TCS SPE; Leica, Buffalo Grove, IL, USA). Sections incubated with no primary antibody served as negative control. It should be noted that immunostaining for KDEL and CHOP was repeated twice on 12 normal and 12 glaucomatous TM tissues to ensure the consistency of data.

Z-stacks were processed in ImageJ (<http://imagej.nih.gov/ij/>); provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). Individual slices were stacked together with the maximum intensity setting. Thus, images shown and analyzed for intensity measurements are projections of multiple slices. To quantify KDEL and CHOP signal intensities in normal and glaucomatous TM, a masked evaluation of each section was used. Relative fluorescence intensities (integrated intensity values) for KDEL and CHOP staining per TM section covering the most of the TM region were obtained using ImageJ software as described previously.³⁰ Relative signal intensities per square TM areas were subtracted with averaged background intensity and are graphically represented in Figures 1B and 1C.

FIGURE 1. Immunostaining for KDEL and CHOP in human TM tissues. (A) Representative immunostaining image of KDEL (red) and CHOP (green) merged with DAPI in age-matched normal (top) and glaucoma (bottom) TM tissues showing increased KDEL and CHOP in the glaucomatous TM. Scale bar: 25 μ m. SC, Schlemm's canal; arrow shows TM. (B, C) Average intensity of KDEL (B) and CHOP (C) per square micrometer area of TM showing increased KDEL and CHOP immunostaining in the glaucomatous TM ($n = 18$ normal, 18 glaucoma, $***P < 0.0001$; t -test).

Western Blotting

Trabecular meshwork protein lysates from four normal and five glaucoma donors (Table 2) were obtained from the laboratory of Sanjoy Bhattacharya (University of Miami) and were processed as described previously.³¹ Trabecular meshwork tissues and cells were lysed with lysis buffer (50 mM HEPES pH 7.0, 200 mM KCl, 1% Triton X-100, 1 mM EGTA, 1 mM MgCl₂, 0.5 mM dithiothreitol [DTT], 10% glycerol) supplemented with Complete Protease Inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA). Cellular proteins were separated on denaturing polyacrylamide gels and then transferred to polyvinylidene fluoride membranes by electrophoresis. Blots were blocked with 5% nonfat dry milk for 1 hour. The blots were then incubated overnight with specific primary antibodies at 4°C. The membranes were washed with Tris-buffered saline/Tween buffer (TBST) and incubated with a corresponding horseradish peroxidase-conjugated secondary antibody (1:10000 dilution in 5% milk; Santa Cruz Biotechnology, Inc.). The proteins were then visualized in Odyssey Fc Imager (LI-COR Biotechnology, Lincoln, NE, USA) using electrochemiluminescence (ECL) detection reagents (Super-Signal West Femto Maximum Sensitivity Substrate; Pierce Biotechnology, Grand Island, NY, USA). To ensure equal protein loading, the same blot was subsequently incubated with actin antibody (Cell Signaling Technology, Inc., Danvers, MA, USA). Due to limited access to TM tissue lysates, a single SDS-PAGE gel was run. The membrane was stripped multiple times and probed with other ER stress antibodies. Quantitation was done using ImageJ software as described previously.²⁰ Antibodies for ATF-4, ERO-1, phosphorylated EIF-2 α , and CHOP

(1:1000 dilution in 5% milk) were purchased from Cell Signaling Technology, Inc. Antibodies for GRP78 and GRP94 (1:500 dilution in 5% milk) were purchased from Santa Cruz Biotechnology, Inc.

XBP-1 Splicing

Total RNA was isolated from four normal TM cell and four glaucomatous TM cell strains. Total RNA from normal and glaucomatous TM cells was reverse transcribed and cDNA was subjected XBP-1 PCR as described previously²⁰ using primers 5-AACTCCAGCTAGAAAATCAGC-3' and 5-CCATGGGAA GATGTTCTGGG-3. Polymerase chain reaction was performed for 25 cycles (94°C, 30 seconds; 55°C, 30 seconds; 72°C, 30 seconds [2 minutes in the final cycle]). Spliced XBP-1 product was expected at 257 base pairs and unspliced XBP-1 product was expected at 283 base pairs.

Statistics

Data are shown as mean $\pm$ SEM. For comparisons between two groups, the unpaired Student's t -test was used (GraphPad Prism, La Jolla, CA, USA). $P < 0.05$ was considered significant.

RESULTS

Immunohistochemical Analysis of ER Stress Markers in Human TM Tissues

We examined ER stress markers in 18 pairs of age-matched normal and glaucomatous TM donor tissues (Figs. 1A–C, 2A–

TABLE 2. List of Normal and Glaucoma TM Tissue Lysates Used for Western Blot

No.	Race	Age	Ocular Disease
1	C	71	NA
2	C	71	NA
3	C	68	NA
4	C	52	NA
5	C	61	Glaucoma and blind OU; unknown eye surgery 1970; wore glasses; went blind in early 20s; scleral buckle OS
6	C	66	Bilateral narrow-angle glaucoma; Tx latanoprost with laser trabeculoplasty; OU; OD synechiae
7	C	70	Bilateral POAG Dx 1/2013 on 0.5% timolol bid; avg. IOP 20; highest IOP 23
8	C	80	Glaucoma/POAG
9	C	76	Glaucoma/POAG

C, Caucasian; NA, not available; OS, left eye, Tx, treated; Dx, diagnosed; OU, both eyes; OD, right eye; bid, twice per day.

C). We immunostained paraffin-embedded anterior segment sections using KDEL and CHOP antibodies in three age-matched groups (70–80 years, $n = 4$; 80–90 years, $n = 11$; 90–100 years, $n = 3$). Figure 1A shows a representative staining with KDEL (red) and CHOP (green) merged with nuclear stain DAPI of human normal (top) and glaucomatous (bottom) TM tissues. Overall, KDEL immunostaining was observed in the TM of normal eyes. However, its level was moderately increased in

the glaucomatous TM (Fig. 1A). Interestingly, there was minimum CHOP immunostaining observed in the normal TM; however, CHOP levels were considerably increased in the glaucomatous TM. Sections incubated with only secondary antibody show some background staining in the ciliary body but minimum background staining in the TM region of normal or glaucoma eyes as shown in the low- and high-magnification images in Figures 2A and 2B, respectively. We observed some

FIGURE 2. (A) Low-magnification images of anterior segment showing increased KDEL and CHOP in the glaucomatous TM. Age-matched normal (left) and glaucoma (right) paraffin-embedded anterior segment tissues stained with KDEL (red) and CHOP (green) antibody and merged with DAPI stain are shown at the top. Sections incubated with only secondary antibody are shown at the bottom, which shows very little background staining. Arrow shows TM. SC, Schlemm's canal, CB, ciliary body, I, iris. Scale bar: 100 μ m. (B) Higher-magnification images showing minimum background staining in normal (left) and glaucomatous (right) TM tissues. Scale bar: 25 μ m. (C) Representative immunostaining for KDEL and CHOP in three age groups of normal and glaucomatous TM tissues. Top: Normal TM (74 years) on the left and glaucoma TM tissues on the right (70 years). Middle: Normal TM donor tissue (82 years) and glaucoma TM donor tissue (85 years). Bottom: Normal TM donor tissue (90 years) and glaucoma TM donor tissue (93 years). Scale bar: 25 μ m.

FIGURE 3. Western blot analysis of ER stress markers in TM tissue lysates. (A) 10 μ g total protein from normal ($n = 4$) and glaucomatous ($n = 5$) TM tissue lysates was electrophoresed in SDS-PAGE gels followed by Western blot analysis for GRP78, GRP94, ERO-1 α , phosphorylated ELF-2 α , ATF-4, and CHOP. Actin was used as a loading control. Samples are loaded from number 1 to 9 in a sequence that is described in Table 2. (B–G) Densitometric analysis of these ER stress markers normalized to loading control in normal and glaucomatous TM. Both GRP78 and GRP94 were increased in the glaucomatous TM but were statistically not significant ($P = 0.087$ for GRP78 and $P = 0.1007$ for GRP94). Markers of chronic ER stress including ERO-1, ATF-4, and CHOP were significantly elevated in the glaucomatous TM ($P = 0.0247$ for ERO-1 α ; $P = 0.0129$ for ATF-4; and $P = 0.0294$ for CHOP; $n = 4$ normal and 5 glaucoma, age 50–80 years).

KDEL staining in the ciliary body of normal TM; however, its level was not changed in the glaucomatous TM (Fig. 2A). Furthermore, we observed that KDEL and CHOP levels were also increased in the glaucomatous TM in all age groups (Fig. 2C). Although both KDEL and CHOP appear to be more pronounced in uveal region of the TM in a representative image, this pattern was not consistently observed in other glaucomatous TM donor tissues. In most cases, we observed increased ER stress markers in the entire TM region.

We further analyzed the intensity of KDEL and CHOP signals in all normal and glaucomatous TM using ImageJ (Figs. 1B, 1C). Average intensity for KDEL was significantly increased in the glaucomatous TM (17.07 ± 2.19 in normal, $n = 18$ vs. 37.81 ± 2.83 in glaucoma, $n = 18$; $P < 0.0001$). Interestingly, there was a 3-fold increase in CHOP staining in the glaucomatous TM (11.84 ± 1.98 in normal, $n = 18$ vs. 33.24 ± 3.89 in glaucoma, $n = 18$; $P < 0.0001$). When intensity differences for KDEL and CHOP between normal and glaucomatous TM were compared, each group showed similar differences. For age group 70 to 80 years, averaged KDEL intensity was 18.5 ± 1.8 in normal and 43.1 ± 7.3 in glaucoma ($n = 4$ each). For age group 80 to 90 years, KDEL intensity was 17.7 ± 3.1 in normal and 35.9 ± 3.8 in glaucoma ($n = 11$ each). In age group 90 to 100 years, KDEL intensity was 12.5 ± 6.6 in normal and 37.3 ± 3.1 in glaucoma ($n = 3$ each). For age group 70 to 80 years, averaged CHOP intensity was 12.1 ± 3 in normal and 30.4 ± 7.1 in glaucoma ($n = 4$ each). For age group 80 to 90 years, KDEL intensity was 12.8 ± 3.1 in normal and 35.8 ± 5.6 in glaucoma ($n = 11$ each). In age group 90 to 100 years, KDEL

intensity was 7.8 ± 0.9 in normal and 27.3 ± 6.9 in glaucoma ($n = 3$ each). Both KDEL and CHOP levels did not change significantly with age of the TM tissue. These data indicate the presence of chronic ER stress in the glaucomatous TM.

Western Blot Analysis of ER Stress Markers in Human TM Tissue Lysates

Since several antibodies for ER stress markers did not work on paraffin-embedded TM sections, we next examined ER stress markers by Western blot analysis of human TM tissue lysates. Western blot analysis demonstrated that GRP78, GRP94, ERO-1 α , ATF-4, and CHOP were increased in the age-matched glaucomatous TM (POAG) tissues compared to normal TM lysates (Figs. 3A–G). Both phosphorylated EIF-2 α (Figs. 3A, 3G) and total EIF-2 α (data not shown) levels were not changed in the glaucomatous TM compared to normal TM tissues. Densitometric analysis of Western blots for ER stress markers normalized to loading control β -actin is shown in Figures 3B through 3G. GRP78 and GRP94 levels were increased in the glaucomatous TM lysates, but the differences were not statistically significant (for GRP78, 0.0022 ± 0.0006 in normal versus 0.016 ± 0.008 in glaucoma, $P = 0.087$; for GRP94, 0.04 ± 0.006 in normal versus 0.2 ± 0.1 in glaucoma $P = 0.1007$; $n = 4$ normal and 5 glaucoma; Figs. 2B, 2C). However, markers of chronic ER stress including ERO-1 α , ATF-4, and CHOP were significantly increased in the glaucomatous TM lysates (for ERO-1 α , 0.0006 ± 0.0002 in normal versus 0.002 ± 0.0008 in glaucoma, $P = 0.0247$; for ATF-4, 0.002 ± 0.001 in normal

FIGURE 4. Examination of ER stress markers in cultured TM cells. (A) Western blot analysis of ER stress markers including GRP78, GRP94, ERO-1 α , ATF-4, and CHOP in three normal and four glaucomatous primary TM cell strains. Actin was used as a loading control. NTM, normal TM cells; GTM, glaucomatous TM cells. (B) Splicing of XBP-1. Confluent normal ($n = 4$) and glaucomatous ($n = 4$) TM cells were grown in serum-free medium for 48 hours and total RNA was subjected to XBP-1 RT-PCR. For dexamethasone treatment, normal TM cells were incubated with dexamethasone (100 nM) for 10 days, and total RNA was subjected to XBP-1 RT-PCR. The resulting products were subjected to 3% agarose gel electrophoresis. Spliced XBP-1 was observed only in dexamethasone-treated sample (unspliced product at 283 base pairs and spliced product at 257 base pairs). N, normal TM; G, glaucomatous TM. Ctl, control NTM treated with vehicle; DEX, NTM treated with 100 nM dexamethasone for 10 days.

versus 0.019 ± 0.005 in glaucoma, $P = 0.0129$; and for CHOP, 0.005 ± 0.002 in normal versus 0.05 ± 0.02 in glaucoma, $P = 0.0294$; $n = 4$ normal and $n = 5$ glaucoma; Figs. 2D–F). No change was observed in phosphorylated pEIF-2 α levels (0.005 ± 0.001 in normal versus 0.007 ± 0.003 in glaucoma, $P = 0.289$). Together, immunostaining and Western blot data indicate the presence of chronic ER stress in the glaucomatous TM.

Examination of ER Stress Markers in Cultured TM Cells

Primary TM cells obtained from glaucoma donors have been shown to maintain their glaucoma phenotypes in culture including increased growth factors,²⁸ extracellular matrix (ECM) proteins,^{30,32} and actin changes.³³ Therefore, we sought to examine whether ER stress markers are also increased in primary TM cells cultured from glaucoma donor eyes compared to normal donors. Western blot analysis of ER stress markers shows that GRP78, GRP94, ERO-1 α , ATF-4, and CHOP were clearly increased in the glaucomatous TM (GTM) cells compared to normal TM cells (Fig. 4A), indicating the presence of chronic ER stress in the glaucomatous TM cells.

Splicing of XBP-1 is considered a classical marker for activation of UPR.⁹ We have previously shown that dexamethasone treatment induces splicing of XBP-1 in TM cells.²⁰ Thus, we further examined whether glaucomatous TM cells activate splicing of XBP-1. As shown in Figure 4B, RT-PCR for XBP-1 shows only unspliced product of XBP-1 in normal and glaucomatous TM cells. As a positive control for XBP-1 splicing, normal TM cells treated with 100 nM dexamethasone show splicing of XBP-1.

DISCUSSION

Increased resistance to aqueous humor outflow through the TM is known to elevate IOP. However, the pathological mechanisms that lead to increased outflow resistance are not understood. Previously, we have shown that increased ER stress in the TM is associated with IOP elevation and that reduction of ER stress rescues glaucoma in mouse models of POAG. In the present study, we further demonstrate the presence of chronic ER stress in the glaucomatous human TM cells and tissues. Glaucomatous TM cells and tissues activate the UPR pathway. Moreover, significantly increased CHOP levels indicate the presence of persistent and unresolved ER stress in glaucomatous TM tissues and cells.

Various physiological conditions can disrupt normal ER homeostasis and cause ER stress. To cope with ER stress, cells activate the cytoprotective UPR pathway. Increased expression of ER chaperones GRP78 and GRP94 indicates the presence of ER stress in the glaucomatous TM cells and tissues. The lack of XBP-1 splicing in GTM cells suggests that glaucomatous cells may not activate protective UPR pathway despite the presence of ER stress. The fact that normal TM cells respond to dexamethasone-induced ER stress by activating XBP-1 splicing suggests that normal TM cells are capable of activating protective UPR. It is also possible that glaucomatous TM cells activate XBP-1 splicing at initial stages of ER stress insult. However, the presence of chronic ER stress over time may diminish protective XBP-1 splicing. In addition to lack of XBP-1 splicing, glaucomatous TM tissues failed to show increased phosphorylation of ELF-2 α (UPR marker), which is involved in general protein translation attenuation and reduction of ER protein load. Lack of XBP-1 splicing and unchanged phosphorylated ELF-2 α suggest that the UPR evoked by the TM in

response to chronic ER stress may be insufficient to normalize ER homeostasis, thus activating chronic ER stress markers such as CHOP, ATF-4, and ERO-1, which are associated with cell dysfunction and death.

Induction of CHOP levels has been shown to be a significant mediator of ER stress-mediated cell dysfunction/death.¹⁴ Importantly, CHOP deletion has been shown to be protective in multiple mouse models.^{20,34,35} We demonstrated that CHOP deletion protects ER stress-mediated IOP elevation in glucocorticoid-induced ocular hypertension.²⁰ Hu et al.³⁵ demonstrated that deletion of CHOP promotes RGC survival in optic nerve crush and ocular hypertensive mouse model. However, it is not clear how CHOP deletion protects from ER stress-mediated cell dysfunction/death. Marciniak et al.¹⁶ demonstrated that CHOP activates ERO-1, which encodes ER oxidase and contributes to reactive oxygen species in ER-stressed cells. The authors further proposed that ER oxidation might induce protein misfolding and accumulation of higher molecular weight proteins. We observed a clear induction of CHOP and ERO-1 α in glaucomatous cells and tissues. It is thus possible that CHOP induces ERO-1 α , which further exacerbates protein misfolding, and the UPR pathway is not sufficient to resolve these misfolded proteins, predisposing cells for cell death/dysfunction. Although CHOP deletion has been shown to protect from ER stress-mediated disease phenotypes, overexpression of CHOP alone does not induce cell death.¹⁵ Induction of CHOP can cause cell death only in ER-stressed cells, suggesting a requirement for other signals. A recent study suggested the roles for ATF-4 and CHOP in ER stress-mediated cell dysfunction and death.¹⁵ A loss of TM cells has been documented in POAG, which may be associated with IOP elevation or the result of chronically elevated IOP.³⁶ Significant induction of the ER-stress proapoptotic proteins CHOP and ATF-4 further suggests the roles of CHOP and ATF-4 in TM cell dysfunction/death and subsequent IOP elevation.

In contrast to our findings that GRP78 is increased in the glaucomatous TM cells (GTM) and tissues, Chai and colleagues³⁷ reported a downregulation of GRP78 in GTM cells compared to normal TM cells. In contrast to Chai and colleagues' study, we demonstrated increased levels of other stable ER stress markers including GRP94, ATF-4, and CHOP in addition to GRP78. There are differences in how primary TM cells were cultured from human patients. We cultured primary TM cells from postmortem donor tissues, whereas Chai and colleagues obtained glaucomatous TM cells from trabeculectomy samples, which were compared to normal TM from postmortem donor tissues. It is possible that cultures obtained from trabeculectomy samples have other cell types present. Most importantly, our study shows increased levels of ER stress markers including GRP94, ERO-1 α , ATF-4, and CHOP in glaucomatous TM tissues ($n = 23$), further strengthening increased ER stress in the glaucomatous TM. Although KDEL and CHOP appear to be colocalizing in the glaucomatous TM tissue (Fig. 1A), a similar trend was not observed in other glaucomatous TM tissues. It is possible that these markers appear to be colocalizing in the representative glaucomatous TM tissues due to close proximity and high levels of expression of these markers. The KDEL antibody recognizes ER resident proteins GRP78 and GRP94, while CHOP resides in cytosol or nucleus. Thus, it is unlikely that these proteins interact with each other.

It is possible that elevated ER stress markers observed in the human glaucomatous TM tissues are a result of chronically elevated IOP. Importantly, we have previously shown that decreasing ER stress can reduce elevated IOP, suggesting that targeting ER stress pathway may facilitate development of disease-modifying glaucoma treatments. The ER stress pathway can be targeted at multiple levels. First, correcting

misfolding of proteins by using a chemical chaperone like PBA can reduce ER stress and protect from IOP elevation as shown in our previous studies.^{17,19,20} A second approach would be strengthening of the UPR pathway such as overexpression of key UPR mediators like spliced XBP-1.³⁸ Spliced XBP-1 has been shown to protect ER stress-mediated RGC death.³⁵ Third, knockdown of CHOP has been shown to be protective in numerous studies including ones on glaucoma.^{13,14,20,34} Future studies aimed at exploring gene therapy to knock down CHOP or overexpress XBP-1 and using therapeutic agents that reduce ER stress will provide a promising approach for treatment of POAG.

Acknowledgments

The authors thank Tien Phan and Sandra Neubauer for assistance in some experiments.

Supported by National Eye Institute Grants EY022077 (GSZ), 2R01EY016242 (AFC), and EY016112 (SB) and funding from the North Texas Eye Research Institute. The Knights Templar Eye Foundation provided financial assistance for some experiments.

Disclosure: **J.C. Peters**, None; **S. Bhattacharya**, None; **A.F. Clark**, Reata Pharmaceuticals (F), Sanofi-Fovea (C), Genzyme-Sanofi (C), ISIS Pharmaceuticals (C); **G.S. Zode**, Oxford BioMedica plc (F)

References

1. Kwon YH, Fingert JH, Kuehn MH, Alward WL. Primary open-angle glaucoma. *N Engl J Med*. 2009;360:1113–1124.
2. Quigley HA. Neuronal death in glaucoma. *Prog Retin Eye Res*. 1999;18:39–57.
3. Quigley HA, Broman AT. The number of people with glaucoma worldwide in 2010 and 2020. *Br J Ophthalmol*. 2006;90:262–267.
4. Quigley HA. Number of people with glaucoma worldwide. *Br J Ophthalmol*. 1996;80:389–393.
5. Leske MC, Connell AM, Wu SY, Hyman LG, Schachat AP. Risk factors for open-angle glaucoma. The Barbados Eye Study. *Arch Ophthalmol*. 1995;113:918–924.
6. Rosenthal J, Leske MC. Open-angle glaucoma risk factors applied to clinical area. *J Am Optom Assoc*. 1980;51:1017–1024.
7. Rohen JW, Witmer R. Electron microscopic studies on the trabecular meshwork in glaucoma simplex. *Albrecht von Graefes Arch Klin Exp Ophthalmol*. 1972;183:251–266.
8. Gabelt BT, Kaufman PL. Changes in aqueous humor dynamics with age and glaucoma. *Prog Retin Eye Res*. 2005;24:612–637.
9. Yoshida H. ER stress and diseases. *FEBS J*. 2007;274:630–658.
10. Schroder M, Kaufman RJ. The mammalian unfolded protein response. *Annu Rev Biochem*. 2005;74:739–789.
11. Ron D, Walter P. Signal integration in the endoplasmic reticulum unfolded protein response. *Nat Rev Mol Cell Biol*. 2007;8:519–529.
12. Nakagawa T, Zhu H, Morishima N, et al. Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. *Nature*. 2000;403:98–103.
13. Oyadomari S, Koizumi A, Takeda K, et al. Targeted disruption of the Chop gene delays endoplasmic reticulum stress-mediated diabetes. *J Clin Invest*. 2002;109:525–532.
14. Oyadomari S, Mori M. Roles of CHOP/GADD153 in endoplasmic reticulum stress. *Cell Death Differ*. 2004;11:381–389.
15. Han J, Back SH, Hur J, et al. ER-stress-induced transcriptional regulation increases protein synthesis leading to cell death. *Nat Cell Biol*. 2013;15:481–490.

16. Marciniak SJ, Yun CY, Oyadomari S, et al. CHOP induces death by promoting protein synthesis and oxidation in the stressed endoplasmic reticulum. *Genes Dev.* 2004;18:3066–3077.
17. Zode GS, Kuehn MH, Nishimura DY, et al. Reduction of ER stress via a chemical chaperone prevents disease phenotypes in a mouse model of primary open angle glaucoma. *J Clin Invest.* 2011;121:3542–3553.
18. Stone EM, Fingert JH, Alward WL, et al. Identification of a gene that causes primary open angle glaucoma. *Science.* 1997;275:668–670.
19. Zode GS, Bugge KE, Mohan K, et al. Topical ocular sodium 4-phenylbutyrate rescues glaucoma in a myocilin mouse model of primary open-angle glaucoma. *Invest Ophthalmol Vis Sci.* 2012;53:1557–1565.
20. Zode GS, Sharma AB, Lin X, et al. Ocular-specific ER stress reduction rescues glaucoma in murine glucocorticoid-induced glaucoma. *J Clin Invest.* 2014;124:1956–1965.
21. Naidoo N. ER and aging—protein folding and the ER stress response. *Ageing Res Rev.* 2009;8:150–159.
22. Hayashi T, Saito A, Okuno S, et al. Oxidative damage to the endoplasmic reticulum is implicated in ischemic neuronal cell death. *J Cereb Blood Flow Metab.* 2003;23:1117–1128.
23. Nishitoh H, Kadowaki H, Nagai A, et al. ALS-linked mutant SOD1 induces ER stress- and ASK1-dependent motor neuron death by targeting Derlin-1. *Genes Dev.* 2008;22:1451–1464.
24. Oh YK, Shin KS, Yuan J, Kang SJ. Superoxide dismutase 1 mutants related to amyotrophic lateral sclerosis induce endoplasmic stress in neuro2a cells. *J Neurochem.* 2008;104:993–1005.
25. Lemaire K, Schuit F. Integrating insulin secretion and ER stress in pancreatic beta-cells. *Nat Cell Biol.* 2012;14:979–981.
26. Anholt RR, Carbone MA. A molecular mechanism for glaucoma: endoplasmic reticulum stress and the unfolded protein response. *Trends Mol Med.* 2013;19:586–593.
27. Izzotti A, Bagnis A, Sacca SC. The role of oxidative stress in glaucoma. *Mutat Res.* 2006;612:105–114.
28. Wordinger RJ, Fleenor DL, Hellberg PE, et al. Effects of TGF-beta2, BMP-4, and gremlin in the trabecular meshwork: implications for glaucoma. *Invest Ophthalmol Vis Sci.* 2007;48:1191–1200.
29. Steely HT, Browder SL, Julian MB, Miggans ST, Wilson KL, Clark AF. The effects of dexamethasone on fibronectin expression in cultured human trabecular meshwork cells. *Invest Ophthalmol Vis Sci.* 1992;33:2242–2250.
30. Medina-Ortiz WE, Belmares R, Neubauer S, Wordinger RJ, Clark AF. Cellular fibronectin expression in human trabecular meshwork and induction by transforming growth factor-beta2. *Invest Ophthalmol Vis Sci.* 2013;54:6779–6788.
31. Bhattacharya SK, Rockwood EJ, Smith SD, et al. Proteomics reveal Cochlin deposits associated with glaucomatous trabecular meshwork. *J Biol Chem.* 2005;280:6080–6084.
32. Tovar-Vidales T, Roque R, Clark AF, Wordinger RJ. Tissue transglutaminase expression and activity in normal and glaucomatous human trabecular meshwork cells and tissues. *Invest Ophthalmol Vis Sci.* 2008;49:622–628.
33. Clark AF, Brotchie D, Read AT, et al. Dexamethasone alters F-actin architecture and promotes cross-linked actin network formation in human trabecular meshwork tissue. *Cell Motil Cytoskeleton.* 2005;60:83–95.
34. Song B, Scheuner D, Ron D, Pennathur S, Kaufman RJ. Chop deletion reduces oxidative stress, improves beta cell function, and promotes cell survival in multiple mouse models of diabetes. *J Clin Invest.* 2008;118:3378–3389.
35. Hu Y, Park KK, Yang L, et al. Differential effects of unfolded protein response pathways on axon injury-induced death of retinal ganglion cells. *Neuron.* 2012;73:445–452.
36. Alvarado J, Murphy C, Juster R. Trabecular meshwork cellularity in primary open-angle glaucoma and nonglaucomatous normals. *Ophthalmology.* 1984;91:564–579.
37. Chai F, Luo R, Li Y, et al. Down-regulation of GRP78 in human glaucomatous trabecular meshwork cells. *Mol Vis.* 2010;16:1122–1131.
38. Calfon M, Zeng H, Urano F, et al. IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. *Nature.* 2002;415:92–96.