POAG is the leading cause of irreversible blindness worldwide. Currently affecting over 60 million individuals, this insidious age- and race-biased optic neuropathy is characterized by a gradual loss of retinal ganglion cell neurons and is projected to impact nearly 80 million people by the year 2020. The prevalence of POAG, the most common form of glaucoma, affects approximately 1.86%, or nearly 2 million individuals, 45 years of age and older in the United States. More recent data using the US Census Bureau’s national population projections (2014–2050) estimate the US population with glaucoma in 2010 at 2.7 million, 2.9 million in 2014, and will increase to 4.3 million and 5.5 million by the years 2032 and 2050, respectively.

Several prospective, randomized, multicenter studies have identified elevated IOP as a primary risk factor for the onset and progression of POAG. The pathophysiology leading to
Mitochondrial-Targeted Antioxidants Attenuate TGF-β2 Signaling

EDUCATED CHANGES IN SMAD-DEPENDENT TRANSCRIPTIONAL ACTIVITY, INCLUDING MARKED REDUCTIONS IN CTGF, COLLAGEN TRANSCRIPTIONAL ISOMERS, AND COLLAGEN PROTEIN EXPRESSION.

METHODS AND MATERIALS

Human Trabecular Meshwork Cell Culture

The use of human material in this study was approved by the Edward Hines Jr. VA Hospital institutional review board. Fresh corneoscleral rims were received in Optisol corneal storage medium from the Illinois Eye Bank (Chicago, IL, USA) at time of corneal transplant and primary human TM cell isolates were prepared using a collagenase-free procedure as we have previously described. The purity of primary TM cell cultures typically exceeded 95% as routinely determined by cell morphology. Primary isolates were passaged (1:5) using trypsin and subsequent primary (secondary normal) TM cell cultures were restricted to less than seven passages. To objectively validate the identity and stability of isolated TM cells, primary human TM cultures were routinely challenged with dexamethasone and analyzed for expression and release of myocilin. Briefly, TM cells were cultured to near confluency in 6-well plates with each well containing 2 mL of either low-glucose (5.6 mM) Dulbecco’s modified Eagles medium (DMEM) media (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 mg/mL streptomycin, 1% amphotericin B (ThermoFisher Scientific, Asheville, NC, USA), and 1% TM cell growth supplement (ScienceCell Research Lab, Carlsbad, CA, USA), or with low-glucose MEM (ThermoFisher Scientific) supplemented with 10% fetal bovine serum, 5% adult bovine serum, essential and nonessential amino acids, 100 U/mL penicillin, 100 mg/mL streptomycin, and 1% amphotericin B. Media was replaced with an equal volume of fresh serum-free low-glucose media containing vehicle (0.25% dimethyl sulfoxide [DMSO]) or dexamethasone (95 nM). After 72 hours, an aliquot (1.2 mL) of conditioned cell culture media was centrifuged at 1000g for 5 minutes to remove nonadherent cells/debris and 1-mL of the resultant supernatant was treated with 10 mL of StrataClean Resin (Agilent Technologies, Santa Clara, CA, USA) to concentrate released myocilin. Resin-treated samples were gently mixed by repeated inversion × 20 minutes at 4°C and resin-captured myocilin collected by centrifugation (20,000g × 5 minutes). Aspirated supernatant was discarded, resin pellets were resuspended with 25 mL of Laemmli sample buffer and heated at 65°C × 10 minutes to elute captured myocilin. Samples were cleared by centrifugation (20,000g × 10 minutes) and an aliquot of the cleared supernatant was qualitatively analyzed by capillary Western immuno-electrophoresis (Wes; Protein Simple, San Jose, CA, USA) for the presence of myocilin using a 1:50 dilution of mouse monoclonal antimycilin primary antibody (EMD Millipore, Temecula, CA, USA) and a ready-to-use horseradish peroxidase conjugated anti-mouse IgG secondary antibody preparation (Protein Simple, San Jose, CA, USA). A prominent doublet (arrows) migrating between 58 and 59 kDa was present in dexamethasone (lanes 4–6), but not vehicle (lanes 1–3), treated samples as shown in Figure 1.

SV40-transformed human TM cells (GTM3) were a generous gift from Alcon Laboratories (Fort Worth, TX, USA) and were cultured in low-glucose (5.6 mM) DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 mg/mL streptomycin, and 1% amphotericin B. In contrast to passaged primary TM cells, conditioned media collected from transformed GTM3 cells and primary human TM cell lines described above contained elevated levels of myocilin even in the absence of a dexamethasone challenge (data not shown). Primary and
transformed human TM cell cultures were maintained at 37\textdegree C under a humidified atmosphere of 5% CO\textsubscript{2}/95% air.

Treatment

Semiconfluent cultures of primary or transformed (GTM3) human TM cells were incubated overnight in serum-free low-glucose media unless noted otherwise. Recombinant human TGF-\(\beta\)2 (Bio-Techne Corporation, Minneapolis, MN, USA) was reconstituted with 4 mM HCl containing 0.1% BSA to a working concentration of 20 \(\mu\)g/mL and aliquots stored at \(-80\)\degree C until use. Stored aliquots were ‘reactivated’ prior to use by diluting stocks 1:1 (vol/vol) with fresh 4 mM HCl-BSA to yield a final concentration of 10 \(\mu\)g/mL. Serum-starved cultures were treated in the absence (vehicle, 2 \(\mu\)M HCl) or presence of TGF-\(\beta\)2 (5 ng/mL). In some experiments, cells were pretreated with SB-431542 (10 \(\mu\)M, a TGF-\(\beta\) type I receptor [TGF\(\beta\)RI]/activin receptor-like kinase 5 [ALK-5] inhibitor) or with mitochondrial-targeted antioxidants XJB-5-131 (10 \(\mu\)M; generous gift from Peter Wipf)\textsuperscript{13} or mitoquinone mesylate (MitoQ, 10 nM; MedKoo Biosciences, Inc., Morrisville, NC, USA).

Quantification of Oxidative Stress

Intracellular ROS were qualitatively and quantitatively measured using oxidation-sensitive fluorogenic dyes CellROX green or 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (Carboxy-H2DCFDA; ThermoFisher Scientific), respectively. For qualitative assessment, TM cells (15,000 per chamber) were cultured to approximately 60% confluency in 8-well Nunc Lab-Tek II chambered glass slides (ThermoFisher Scientific), serum-starved overnight, and subsequently treated as described above. CellROX green (5 \(\mu\)M) was added to the final 30 minutes of treatment. Dye-loaded cells were fixed by immersion in 3.7% buffered paraformaldehyde (pH 7.4) at 23\degree C for 15 minutes. Fixed cells were rinsed, air dried, and mounted with Fluoroshield containing 4',6-diamidino-2-phenylindole (DAPI). Cells were imaged using a Leica TCS SPE confocal microscope (Leica Microsystems, Buffalo Grove, IL, USA) and LAS-X imaging suite. To minimize photoactivation artifact, cells fields were imaged under identical confocal conditions using identical software settings. For quantitative assessment, TM cells were cultured in 96-well plates to near confluency and serum-starved overnight. Media was removed, and cells were preincubated 30 minutes at 37\degree C with prewarmed Hanks’ balanced salt solution supplemented with carboxy-H2DCFDA (5 \(\mu\)M). Dye preloaded cells were subsequently incubated in fresh serum-free media for an additional 30 minutes at 37\degree C prior to treating as described above. The 2′,7′-dichlorofluorescein diacetate fluorescence was quantified using a Labtech FLUOstar Optima luminometer (BMG Labtech, Cary, NC, USA) at 488-nm excitation/520-nm emission wavelengths.

Real-Time RT-PCR

Total RNA from TM cells was isolated using TRizol reagent (ThermoFisher Scientific) and 1 \(\mu\)g was reverse-transcribed using iScript Supermix (Bio-Rad Laboratories, Hercules, CA, USA). cDNA sequences were amplified by real-time (iQ SYBR Green Supermix; Bio-Rad) quantitative PCR using a 48-well CFX Connect PCR detection system with published human-specific primer pairs (Table 1). Human-specific glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primer pairs were used as a reference control. For each sample, the specificity of the real-time reaction product was determined by melting curve analysis. Reaction efficiencies were typically >90%. The endogenous expression of GAPDH was unaltered by drug treatment (data not shown). Relative fold changes in gene expression in each sample were therefore normalized to expressed levels of GAPDH.

Immunoblot Analysis

Human TM cells, treated as described above, were rinsed with ice-cold PBS and lysed by the addition of lysis buffer containing 20 mM Tris HCl (pH 7.5), 150 mM NaCl, 1% Triton X100, and 1

![Figure 1](https://example.com/figure1.png)
mM EDTA supplemented with a cocktail of protease (Roche Diagnostics Corp., Indianapolis, IN, USA) and phosphatase (Sigma Aldrich) inhibitors. Lysates were clarified by centrifugation (20,000g × 10 minutes at 4°C). To quantify secreted proteins, aliquots of conditioned media from treated cells were concentrated by centrifugal filtration (10-kDa cutoff; Amicon; Millipore, Bedford, MA, USA). Clarified cell lysates and concentrated condition media were stored at −80°C until use. Protein concentrations in clarified cell lysates and concentrated conditioned media were determined by the BCA method (Thermo Scientific) using BSA as a standard. Proteins (10-μg protein per lane) in cell lysates or conditioned media were resolved by 4% to 20% SDS-PAGE gel electrophoresis either under reducing/denaturing (for Smads) or reducing (for collagens) conditions and transferred overnight onto nitrocellulose (Smads) or polyvinylidene difluoride (collagens) membranes as previously described. Membranes were blocked with 5% Carnation non-fat dried milk (Nestle, Minneapolis, MN, USA) in 10 mM Tris buffer (pH 7.4) containing 0.1% Tween 80 × 1 hour at 23°C and incubated overnight at 4°C in the presence of a 1:1000 dilution of rabbit monoclonal anti-Smad2 (clone 86F7), anti-phospho-Smad2 (clone 138D4; Ser465/467), anti-Smad3 (clone C67H9), anti-phospho-Smad3 (clone C25A9; Ser423/425) primary antibodies (Cell Signaling Technology, Danvers, MA, USA), or with a 1:1000 dilution of sheep polyclonal anti-collagen IV (ab6586; Abcam, Cambridge, MA, USA) or a 1:8000 dilution of rabbit polyclonal anti-collagen I (AF6220-SP; R&D Systems) secondary antibody, respectively. Immunostained membranes were washed in Tris-Tween buffer and incubated 1 hour at 23°C in the presence of a 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (ThermoFisher Scientific), or a 1:1000 dilution of rabbit monoclonal anti-collagen IV (ab6586; Abcam, Cambridge, MA, USA) primary antibodies, as indicated. Immunostained membranes were washed in Tris-Tween buffer and incubated × 1 hour at 23°C in the presence of a 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Labs, Inc., West Grove, PA, USA) or a 1:1000 dilution of horseradish peroxidase-conjugated donkey anti-sheep IgG (R&D Systems) secondary antibody, respectively. Immunostained proteins were visualized by enhanced chemiluminescence using Supersignal West Pico chemiluminescent substrate (ThermoScientific, Rockford, IL, USA).

Immunocytochemistry

Passaged human TM cells were cultured to near confluency in 8-well chambered slides, serum-starved overnight, and subsequently treated as described above. Treated TM cells were rinsed with ice-cold PBS (pH 7.4) and fixed by immersion in 4.0% buffered paraformaldehyde (pH 7.4) × 15 minutes at 23°C. Fixed cells were permeabilized with 0.05% Triton X-100 in PBS × 15 minutes at 23°C, blocked with 5% BSA in PBS (pH 7.4) for 1 hour at 23°C, and subsequently incubated overnight at 4°C in the presence of a 1:500 dilution of either mouse monoclonal anti-collagen I (ab90395; Abcam) or rabbit polyclonal anti-collagen IV (ab6586; Abcam) primary antibody, as indicated. Immunostained cells were washed in PBS, co-incubated for 1 hour at 23°C with a 1:1000 dilution of either AlexaFluor 488-conjugated goat anti-mouse IgG or AlexaFluor 594-conjugated goat anti-rabbit IgG secondary antibody (ThermoFisher Scientific), respectively, in the presence of DAPI (NucBlue; ThermoFisher Scientific). Washed cells were air dried, and mounted with Aqua Polycl Mount (PolySciences, Inc., Warrington, PA, USA). Cell immunofluorescence was captured using a Leica TCS SPE confocal microscope and imaging suite. Images were Z-stacked at 1-μm steps acquired from below the base of the cells to the top of the cells. Relative fluorescence intensities of representative immunostained fields were quantified using imaging software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

Measurements of Smad-Binding Element (SBE)-Luciferase Activity

Transformed (GT3M) human TM cells were cultured in 96-well plates (20,000 cells per well) to near confluency and reverse transfected with 50 ng of luc2P/SM/EHygro plasmid (Promega, Madison, WI, USA) using Lipofectamine 2000 transfection reagent (ThermoFisher Scientific) according to the manufacturer’s protocol. The luc2P/SM/EHygro plasmid is a pGL4.48 6052 bp vector containing SBE-binding response elements upstream of firefly luciferase 2P (1-hour half-life) gene. To control for potential variations in transfection efficiency, cells were co-transfected with thymidine kinase-RENilla luciferase. Transfected TM cells were subsequently cultured in serum-free media overnight and treated as described above. Following 24 hours of treatment, cells were lysed, and lysates were assayed for luciferase activity according to manufacturer’s protocol (Promega). Relative changes in luciferase activity were quantified by luminometry using a Labtech FLUOstar Optima luminometer (BMG LabTech, Inc., Cary, NC, USA).

Quantification of Total TGF-β2 Content in Porcine Anterior Segment Perfusates

Porcine globes were obtained fresh from a local abattoir within 1 to 2 hours of death by electrocution. Anterior segments were aseptically prepared within 4 to 6 hours of procurement and perfused with serum-free DMEM in pairs at a constant flow rate of 4.5 μL/min as previously reported. Following stabilization after a 24-hour washout period, pretreatment baseline aliquots (1.0 mL) of perfusates were collected and stored at −80°C. Stabilized porcine segments were subsequently perfused with vehicle (0.25% DMSO) or with XJB-5-131 (10 μM) for up to an additional 48 hours, at which time posttreatment aliquots (1.0 mL) of perfusates were collected and stored at −80°C. The content of total TGF-β2 protein released from and recovered in collected perfusates, as well as cell culture media, was determined using a commercially available ELISA kit (R&D Systems) according to the manufacturer’s protocol.

Statistical Analysis

Data are expressed as the mean ± SD of N observations unless noted otherwise. Statistical significance of parametric data was determined by Student’s t-test. Significance between multiple experimental groups was determined by one- or two-way ANOVA with a Dunnett’s multiple comparison post hoc analysis. In each case, P < 0.05 was considered statistically significant.

RESULTS

Mitochondrial-Targeted Antioxidants Attenuate TGF-β2–Mediated Oxidative Stress

Primary or transformed human TM cells conditioned overnight in serum-free low glucose media responded robustly to a TGF-β2 challenge (5 ng/mL, 2 hours) by significantly increasing the intracellular content of ROS as quantified by CellROX green fluorescence (Fig. 2A). Pretreating TM cells with SB-435142 (10 μM, a TGF-β type 1 receptor inhibitor) or with mitochondrial-targeted antioxidants (10 nM, XJB-5-131 or MitoQ) completely prevented TGF-β2-mediated increases in intracellular ROS (Fig. 2A). Quantifying relative changes in intracellular levels of ROS using a distinctly different oxidation-sensitive fluorescent dye, carboxy-H2DCFDA, produced quantitatively similar findings. Carboxy-H2DCFDA preloaded TM cells re-
sponded to TGF-β2 (5 ng/mL, 2 hours) by increasing the content of intracellular ROS approximately 2-fold (Fig. 2B). Similar with that observed using CellROX, pretreating TM cells with SB-431542 or with mitochondrial-targeted antioxidants (XJB-5-131 or MitoQ) prevented TGF-β2–mediated increases in intracellular ROS (Fig. 2B).

Mitochondrial-Targeted Antioxidants Attenuate TGF-β2–Mediated Increases in Collagens 1α1 and 4α1 mRNA Expression

In agreement with previous studies,42,43 TGF-β2 (5 ng/mL, 16 hours) also elicited a marked (~5–25-fold) and statistically significant increase in the content of connective tissue growth factor (CTGF), collagen 1α1 (COL1A1), and collagen 4α1 (COL4A1) mRNA expression in conditioned primary or transformed human TM cells (Fig. 4). Pretreating TM cells with SB-431542 or with mitochondrial-targeted antioxidants (XJB-5-131 or MitoQ), however, prevented TGF-β2–mediated increases in CTGF, COL1A1, and COL4A1 mRNA content (Figs. 4A, 4B). In some cases, treating TM cells with SB-431542 or antioxidants alone elicited marked reductions in the baseline levels of CTGF, COL1A1, or COL4A1 mRNA content (data not shown).
Mitochondrially-targeted antioxidants attenuate TGF-β2 Smad-dependent signaling. Serum-starved confluent cultures of (A) primary or (B) transformed TM cells were pretreated (2 hour, 37°C) with vehicle (0.1% DMSO), SB-431542 (10 μM), XJB-5-131 (10 μM), or MitoQ (10 nM), and subsequently incubated for an additional 24 hours (37°C) in the absence or presence of TGF-β2 (5 ng/mL), as indicated. (A, B) Representative immunoblots of phospho- or total Smad 2/3 proteins present in lysates from treated TM cells. Data shown are the means ± SD (N = 3 separate experiments) of digitized band densities, expressed as a ratio of phosphorylated/total Smad proteins. (C) Relative luminescence from Smad-binding element (SBE)-dependent luciferase activity. Data shown are the means ± SD (N = 4) from a single experiment, representative of two additional experiments.

**Figure 3.**
Mitochondrial-Targeted Antioxidants Attenuate TGF-β2–Mediated Increases in Collagens I and IV Subunit Isoform Protein Expression

Consistent with the observed increases in collagen isoform mRNA expression, primary TM cells conditioned overnight in serum-free, low-glucose media and subsequently challenged with TGF-β2 (5 ng/mL, 72 hours) elicited a marked (~1.5- to 2-fold) increase in collagen I and collagen IV protein expression as determined by immunocytochemistry (Fig. 5A). As we have previously reported,44 TGF-β2 also elicited a significant increase in phalloidin-positive filaments (F-actin stress fibers). TGF-β2–mediated increases in actin stress fiber formation and in collagens I and IV protein expression were prevented when assayed in the presence of SB-431542, XJB-5-131, or MitoQ (Figs. 5A). When assayed by immunoblot under reducing non-denaturing conditions, similar qualitative increases in the content of collagens I and IV proteins were seen in lysates of TM cells responding to a TGF-β2 challenge (Fig. 5B, lysates). Similarly, the content of collagens I and IV proteins present in concentrated TM cell culture media was markedly increased in response to a TGF-β2 challenge (Fig. 5B, media). Pretreating primary TM cells with SB-431542 prevented TGF-β2–mediated increases in collagen I and IV content in cell lysates and in culture media (Fig. 5B). While pretreating TM cells with XJB-5-131 or MitoQ only partially prevented TGF-β2–mediated increases in lysate collagen protein content, these antioxidants attenuated TGF-β2–mediated release of collagen protein isoforms into the culture media (Fig. 5B).

Mitochondrial-Targeted Antioxidants Alone Attenuate Endogenous Expression and Release of TGF-β2

As we have previously reported, human TM cells are capable of serving as an endogenous source of biologically active TGF-

![Figure 4](https://iov.s.arvojournals.org/article-pdf/60/10/3619/3259733 iovs_19-3619.pdf)

**Figure 4.** Mitochondrial-targeted antioxidants attenuate TGF-β2–mediated increases in CTGF, collagen 1α1, and collagen 4α1 mRNA content. Serum-starved confluent cultures of (A) primary or (B) transformed TM cells were pretreated (2 hours, 37°C) with vehicle (0.1% DMSO), SB-431542 (10 μM), XJB-5-131 (10 μM), or MitoQ (10 nM), and subsequently incubated for an additional 24 hours (37°C) in the absence or presence of TGF-β2 (5 ng/mL), as indicated. Shown are GAPDH-normalized changes in CTGF, collagen 1α1, and collagen 4α1 mRNA content from treated (A) primary or (B) transformed TM cells, as indicated. Data are the means ± SD (N = 4) from a single experiment. *P < 0.05; **P < 0.01, compared with vehicle controls; one-way ANOVA with Dunnett’s post hoc analysis. In all but one case, TGF-β2–mediated responses from cells pretreated with SB-431542 or with antioxidants were not significantly different from vehicle-treated controls. Pretreatment with mitoquinone was not as effective as XJB-5-131 at attenuating TGF-β2–mediated changes in Smad2 phosphorylation.
β2. To determine whether mitochondrial-targeted antioxidants alone alter the endogenous expression of TGF-β2, transformed TM cells were cultured in the presence of XJB-5-131 (10 μM) for 24 hours. A marked (~80%) reduction in the endogenous content of TGF-β2 mRNA expression was observed in cells treated with XJB-5-131 (Fig. 6A). The absolute content of total TGF-β2 protein secreted by TM cells was also significantly reduced by XJB-5-131 treatment (Fig. 6B). To assess the physiological relevance of these in vitro findings, stabilized porcine anterior segments were perfused with vehicle (0.25% DMSO) or with XJB-5-131 (25 μM) for 48 hours and total TGF-β2 protein released into the perfusate was quantified by ELISA. Segments perfused with XJB-5-131 showed a marked 47% reduction in recovered total TGF-β2 protein. By comparison, vehicle perfused segments exhibited a 12% reduction in released total TGF-β2 protein (Table 2).

**DISCUSSION**

In this study, the multifunctional pro-fibrotic cytokine TGF-β2 is shown to elicit a marked increase in intracellular ROS in both transformed and primary human TM cells, as quantified using two distinct oxidation-sensitive fluorogenic dyes. Pretreating TM cells with mitochondrial-targeted antioxidants attenuated TGF-β2-mediated changes in ROS and in Smad-dependent canonical signaling, including marked reductions in actin stress fiber formation, as well as reductions in CTGF and collagen isoform gene and protein expression. Collectively, these findings suggest that mitochondrial-targeted antioxidants exhibit potential as a novel strategy by which to attenuate TGF-β2-mediated remodeling of the ECM within the TM.

Numerous clinical and experimental studies now support a pivotal role for oxidative stress in the early development and progression of several retinal neurodegenerative diseases, including POAG. Produced as a natural byproduct of enzymatic NADPH oxidase (NOX) and oxidative metabolism, highly reactive superoxide anions (O₂⁻) and hydroxyl (OH) free radicals are largely generated in excess amounts within the mitochondrial respiratory chain as a consequence of compromised or otherwise altered cellular respiration. Membrane permeant hydrogen peroxides can further react with the reduced form of intracellular iron (Fe²⁺) to generate additional amounts hydroxyl free radicals via the Fenton reaction. These reactive oxidants are thought to exert their deleterious effects intracellularly by chemically altering nucleic acids, polyunsaturated lipids, proteins, as well as carbohydrates.

Oxidative stress, in general, is defined as an imbalance between natural intracellular antioxidant defense mechanisms and metabolically generated ROS and has been reported to elicit pronounced functional and morphologic impairments in retinal pigment epithelial cells, endothelial cells, and retinal ganglion cell neurons. Cells of the TM are no exception and...
Mitochondrial-Targeted Antioxidants Attenuate TGF-β2 Signaling

Figure 6. Mitochondrial-targeted antioxidants attenuate endogenous expression and release of TGF-β2. Serum-starved confluent cultures of transformed TM cells were incubated for 24 hours at 37°C in the absence (vehicle, 0.1% DMSO) or presence of XJB-5-131 (10 μM), as indicated. Data shown are the means ± SD (N = 6 wells from 2 separate experiments) of relative changes in (A) GAPDH-normalized TGF-β2 mRNA content and (B) secreted total TGF-β2 recovered from conditioned cell culture media. *P < 0.05; unpaired Student’s t-test.

have proven to be exquisitely vulnerable to oxidative injury. Several lines of additional evidence suggest that POAG may arise as a consequence of age-related mitochondrial dysfunction. A precedence for mitochondrial-dependent cellular energy dysfunction is not, however, unique to POAG. Mitochondrial-dependent pathologies are now overwhelmingly implicated in the genesis of numerous ocular and nonocular neurodegenerative disorders.

Profibrotic disorders involving excessive TGF-β signaling are, similarly, not unique to POAG. Pulmonary fibrosis, for example, is one such disease where oxidative stress and aberrant TGF-β signaling, two seemingly unrelated pathogenic mechanisms, have been causally linked. Mitochondrial-generated ROS have been shown to play a penultimate role in TGF-β-mediated pulmonary fibrosis. Advancements from pulmonary and other fields collectively demonstrate that (1) TGF-β alone induces the generation of mitochondrial ROS from complex III of the electron transport chain, (2) mitochondrial generated ROS are required for TGF-β-induced gene expression downstream of TGF-βRI receptor activation, (3) upstream of Smad2/3 phosphorylation and nuclear translocation, (4) TGF-β-induced transcription of NADPH oxidase 4 requires mitochondrial generated ROS, thereby establishing a feed-forward reciprocal loop leading to increased production of intracellular ROS, (5) fibroblasts from patients with lung fibrosis produce more mitochondrial ROS and a higher level of profibrotic gene expression in response to TGF-β than lung fibroblasts from normal donors, and (6) blocking mitochondrial ROS generation with a mitochondrial-targeted antioxidants markedly attenuates TGF-β-induced profibrotic gene expression.

What remains to be established, and is addressed in part by this study, is whether a similar feed-forward reciprocal relationship between TGF-β2 signaling and mitochondrial-generated ROS occurs in human TM cells. We and others have previously reported that cultured human TM cells respond robustly to a TGF-β2 challenge by promoting F-actin stress fiber formation and by eliciting the expression of ECM components. Human TM cells are also capable of constitutively expressing and releasing biologically active TGF-β2, serving as a localized source of this profibrotic cytokine. Consistent with the reported POAG-associated pathogenicity of TGF-β2, we now show that this cytokine is capable at physiologic levels of eliciting a robust and sustained increase in oxidative stress in human TM cells. In agreement with previous studies, TGF-β2-mediated production of oxygen free radicals did not appear to alter cultured TM cell viability as quantified by MTT and LDH release assays (data not shown). TGF-β2 did, however, elicit measurable increases in TGF-β type I receptor (Alk5)-dependent phosphorylation of regulated Smads 2/3 and Smad-binding element activity, consistent with activation of the canonical signaling pathway. Pretreating cultured TM cells with mitochondrial-targeted antioxidants not only prevented TGF-β2-mediated increases in oxidative stress, but also unexpectedly attenuated canonical signaling by this cytokine. The mechanism(s) by which oxidative stress enhances TGF-β2 canonic signaling remains poorly understood but may involve inhibition of redox sensitive protein phosphatases (PP1, PP2A, or PTEN). These data raise the intriguing possibility that TGF-β2-generated intracellular ROS enable or facilitate TGF-β2 canonic signaling in human TM cells.

The functional relevance of this observation is hallmarked by the ability of mitochondrial-targeted antioxidants to significantly attenuate TGF-β2-mediated increases in F-actin stress fiber formation and TGF-β2-mediated increases in...
Mitochondrial-Targeted Antioxidants Attenuate TGF-β2 Signaling

expression of CTGF and collagens I and IV ECM constituents. Given the well-established ability of TGF-β2 to increase IOP, it presumably through remodeling of the ECM, these data would seem to support a central role of mitochondrial-generated ROS in TGF-β2-mediated decreases in outflow facility and increased IOP in POAG. In agreement with this thesis, the mitochondrial-targeted antioxidant XJB-5-131 is shown to significantly attenuate the endogenous expression and release of TGF-β2 from transformed human TM cells. In addition, perfusing stabilized porcine anterior segments with XJB-5-131 reduced the levels of total TGF-β2 present in segment perfusates. These data not only support endogenous expression and release of TGF-β2 within the TM but also suggests that it is possible to alter, in vivo, endogenous TM levels of this profibrotic cytokine.

Whether mitochondrial-generated ROS are causal to, or a consequence of, the disease process remains unclear. If causal, one may anticipate a beneficial clinical effect of supplementary antioxidant therapy on development/progression of POAG. Epidemiologic studies conducted evaluating the impact of supplementary intake of antioxidants (vitamins A, C, or E) at altering the risk59 or prevalence60 of POAG has been, however, largely unsupportive. An early study by Kang et al.59 failed to observe any strong associations between vitamin A consumption and the risk of POAG. These investigators did report a statistically significant, albeit isolated, protective effect in the third quintile of total vitamin C intake on odds risk of glaucoma. A study by Coleman et al.61 similarly failed to find an association between glaucoma and vitamin nutrients. Interestingly, in the Coleman et al.61 study, consumption of certain foods, such as collards, kale, and carrots, appeared to be protective against glaucoma, unrelated to the intake of vitamins A, C, or E. A more recent study by Wang et al.62 also failed to find an association between supplementary consumption of vitamins A or E and glaucoma prevalence. In this latter study, however, vitamin C supplementation was found to be associated with decreased odds ratio (0.47, 95%CI 0.23–0.97) for glaucoma. No significant differences in ascorbic acid content, however, were present in AH of POAG patients compared with AH from healthy controls.63 In contrast, ascorbic acid levels present in AH of patients with exfoliation syndrome or POAG were substantially lower than in AH of patients with cataract.35 In some64,65 (but not all66–68) studies, administration of ascorbic acid to glaucoma patients markedly lowered IOP.

While these and other related clinical trials would argue against the effectiveness of supplementary antioxidant therapy as an effective adjuvant for the management of POAG, a potential limitation of these trials may be attributed to (1) the route of antioxidant administration rather than a lack of antioxidant effect, and/or the (2) type of antioxidants used as previous studies have shown that mitochondrial-targeted antioxidants offer greater protection against oxidative stress compared with nontargeted antioxidants.69–71 Based on the findings from the current in vitro study, we propose that mitochondrial-targeted antioxidants might serve as a useful adjunct to conventional proven IOP-lowering therapies. Targeted delivery of antioxidants with the use of nanotechnology is emerging as a viable alternative strategy by which to deliver such adjuncts directly to the site of greatest resistance (the JCT) and improve therapeutic efficacy in POAG.

Acknowledgments

The authors thank Charles Bouchard, MA, MD, for assistance with procuring corneoscleral rims for preparation of primary human TM cells, Joo Hong for technical assistance, and Matthew Collins for conducting preliminary studies with XJB-5-131.

Supported by grants from the Department of Veterans Affairs (EBS: 1I21RX001593, 1I01BX003938; Washington, DC, USA), Glaucoma Research Foundation Shaffer Grant (EBS: San Francisco, CA, USA), the Illinois Society for the Prevention of Blindness (EBS: Chicago, IL, USA), the Richard A. Perritt, M.D. Charitable Foundation (EBS: SK; Maywood, IL, USA), The Glaucoma Foundation (SK: New York, NY, USA), and the Dr. John P. and Theresa E. Mulcahy Endowed Professorship (SK; Maywood, IL, USA).

Disclosure: V.R. Rao, None; J.D. Lautz, None; S. Kaja, None; E.M. Foecking, None; E. Lukács, None; E.B. Stubbs Jr, None

References

Mitochondrial-Targeted Antioxidants Attenuate TGF-β2 Signaling


55. Cucoranu I, Clempus R, Dikalova A, et al. NAD(P)H oxidase 4 mediates transforming growth factor-beta1-induced differen-


