Glaucoma

Anti-Connective Tissue Growth Factor Antibody Treatment Reduces Extracellular Matrix Production in Trabecular Meshwork and Lamina Cribrosa Cells

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PURPOSE. We have previously demonstrated elevated levels of connective tissue growth factor (CTGF/CCN2) in the aqueous humor (AqH) of pseudoexfoliation glaucoma (PXFG) patients when compared with cataract controls. Furthermore, there is a significant trabecular meshwork (TM) and lamina cribrosa (LC) fibrotic phenotype associated with glaucoma, possibly driven by CTGF. The purpose of this study was to investigate the potential of anti-CTGF immunotherapy in glaucoma.

METHODS. Primary TM and LC cells were cultured from human donors with (GTM/GLC) and without (NTM/NLC) primary open angle glaucoma (POAG). Aqueous humor samples from PXFG, POAG, and control cataract patients were applied to N/GTM and N/GLC cells in the presence or absence of a therapeutic, humanized monoclonal anti-CTGF antibody FG-3019 (10 μg/mL). Hydrogen peroxide (H2O2) was also used as a stimulus. Expression of fibrotic genes (fibronectin-1, fibrillin-1, CTGF, collagen type 1 α1, and α-smooth muscle actin) was assessed by q-PCR. Protein expression of collagen 1A1 and α-smooth muscle actin was examined in N/GTM cells by SDS-PAGE. The modulatory effect of FG-3019 (10 μg/mL) and IgG (10 μg/mL) were also assessed.

RESULTS. Treatment of cells with AqH from PXFG and POAG patients and H2O2 induced a significant (P < 0.05) increase in expression of profibrotic genes, which was significantly reduced by pretreatment with FG-3019 (P < 0.05). FG-3019 also reduced expression of α-smooth muscle actin and collagen 1A1 protein expression in N/GTM cells.

CONCLUSIONS. FG-3019 is effective in blocking extracellular matrix production in TM and LC cells, thus supporting a role for the use of anti-CTGF immunotherapy in the treatment of glaucoma.

Keywords: glaucoma pharmacology, extracellular matrix, lamina cribrosa, trabecular meshwork

Glaucoma is the second leading cause of irreversible blindness worldwide, though to affect 60 million people.1,2 In the Western world, glaucoma affects 1% to 2% of the population over the age of 40 and the prevalence rises to 5% of those aged 70 years and older. It is a chronic progressive optic neuropathy with characteristic extracellular matrix (ECM) changes in the optic nerve head (ONH) and subsequent visual field defects. The main risk factor for onset and progression of the disease is raised intraocular pressure (IOP),3–7 which is a result of obstruction to aqueous humor (AqH) outflow at the level of the trabecular meshwork (TM). Lowering of IOP is currently the only therapeutic approach available, which does not address the underlying ECM/fibrotic pathology.

The connective tissue changes in primary open angle glaucoma (POAG) affects the TM and the lamina cribrosa (LC) and may result from a common defect in these two cell types. It has been hypothesized that the TM and the LC are biochemically similar tissues and that the cells cultured from the two are very similar.8–15 Our group and others have previously examined the fibrotic phenotype associated with glaucoma in the LC and TM regions, including increased expression of collagen type 1,9,15,16

In glaucoma, the LC undergoes thickening17 and posterior migration18 in the early stages of the disease process, and later undergoes shearing and collapse of the LC plates finally leading to a thin fibrotic connective tissue structure/scar.19 Similar to the LC, the TM of patients with POAG is characterized by the buildup of ECM material20 and this accumulation eventually results in increased outflow resistance with subsequent elevated IOP. Pseudoexfoliation (PXF) syndrome is currently the single most important identifiable risk factor for open-angle glaucoma.21 It is an age-related generalized disorder of the ECM characterized by the production and progressive accumulation of fibrillar material (such as fibronectin [FN] and fibrillin-1 [FB])
in ocular tissues and in the connective tissue portions of the various visceral organs.22–23

There has been increasing interest in the role of proteins, including transforming growth factor beta (TGF\(\beta\)) and connective tissue growth factor (CTGF), in AqH homeostasis leading to raised IOP and glaucoma.24–25 Junglas et al.26 have used a transgenic mouse model to show that CTGF expressed in the AqH elevates IOP, which is associated with TM actin cytoskeleton modification. CTGF is a matricellular protein in that it interacts with and is induced by TGF\(\beta\) and it is through CTGF that TGF\(\beta\) mediates some of the downstream effects on proliferation, migration, and ECM production.27,28 TGF\(\beta\) alters ECM production and turnover in both the LC and TM and has been shown in numerous studies to play a role in ocular wound healing.29,30 while its role in the pathogenesis of glaucoma is also well documented.31–33 Several studies have reported elevated AqH levels of TGF\(\beta\)1 in POAG patients.34,35 and TGF\(\beta\)1 in PXFG patients.36 Our studies have shown that TGF\(\beta\)1 has an effect on global gene expression profiles, especially profibrotic ECM genes in nerve head LC cells.37 We have previously shown that the CTGF level in the AqH of patients with PXFG was significantly higher than in both POAG and normal control subjects.37,38 It appears that coordinate expression of TGF\(\beta\) and CTGF is a normal feature of wound healing. However, pathological fibrosis is often attributed to uncontrolled matrix deposition, perhaps mediated by a CTGF-enriched microenvironment. This has therefore focused attention on CTGF as a possible therapeutic target while avoiding the pleiotropic effects of TGF\(\beta\) inhibition.

The generation of free radicals may be partially responsible for changes in the physiology and morphology of the outflow pathway and associated loss of TM tissue function in glaucoma.39–41 Cells in the TM are subjected to chronic oxidative stress through reactive oxygen species (ROS) generation by normal metabolism and those present in the aqueous humor.42 Oxidative stress can affect cytoskeletal structure and cell-matrix interactions in the TM.43 Mitochondrial production of ROS have been shown to be elevated in TM cells from glaucoma donors44 and we have demonstrated a similar elevation of ROS and a compromised antioxidant potential production in LC cells from glaucoma donors.44 In our current study, we directly address the ongoing fibroct ECM pathology in the TM and LC regions by using a therapeutic anti-CTGF antibody (FG-3019; FibroGen, Inc., San Francisco, CA), previously shown to have therapeutic benefits in models of fibro-proliferative renal disease.45 Our data demonstrate that exposure of TM and LC cells to AqH from both PXFG and POAG patients as well as H\(_2\)O\(_2\) (oxidative stress) induced a significant increase in expression of fibrotic ECM genes (fibronectin, fibrillin-1, CTGF, collagen 1, and \(
\alpha\)-smooth muscle actin [\(\alpha\)-SMA]), which was significantly reduced by pretreatment with FG-3019. The ability of FG-3019 to reduce protein expression of collagen IAI and \(\alpha\)-smooth muscle actin in TM cells was also demonstrated. Thus, anti-CTGF immunomodulation offers a potential novel therapeutic disease modifying strategy for PXFG and POAG.

**METHODS**

**Lamina Cribrosa, Trabecular Meshwork, and ARPE-19 Cell Culture**

Human glial fibrillary acidic protein (GFAP) negative lamina cribrosa cells were obtained from donor eyes with and without a history of glaucoma (POAG) as previously characterized.46 Donors were as follows: NLC215, 67-year-old male; NLC176, 72-year-old male; NLC186, 80-year-old female; GLC201, 74-year-old male; GLC358, 83-year-old male; and GLC428, 68-year-old female. Average age of donors was as follows: normal = 73 ± 4 years and glaucoma = 75 ± 6 years, \(P \leq 0.05\). Cultures were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum, 2 mM L-glutamine, 10,000 units/mL penicillin, and 10 mg/mL streptomycin (Sigma-Aldrich, Wicklow, Ireland). Cultures used in experimental procedures were between passages 4 and 8.

Human TM cells were isolated from carefully dissected human TM tissue explants derived from donor eyes and characterized as previously described.14–17–52 Donors were as follows: NLM 160, 73-year-old male; NTM 416, 78-year-old male; NTM 444, 85-year-old male; GTM 460, 77-year-old male; GTM 686, 71-year-old male; GTM 473, 86-year-old male. Average age of donors was as follows: normal = 78.6 ± 4.9 years and glaucoma = 78 ± 6 years, \(P \geq 0.05\). Cells were cultured in DMEM supplemented with 2 mM L-glutamine, sodium pyruvate, 10% fetal bovine serum, 50 U/mL penicillin and 50 \(\mu\)g/mL streptomycin. Cells are used between passages 4 and 8.

Human retinal pigment epithelial cells (ARPE-19s) were cultured in classical medium (DMEM F12 Ham’s; Sigma- Aldrich, CA) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum, 2 mM L-glutamine, and 5 mg/mL penicillin/streptomycin.

**Reagents**

Recombinant (Rh) human CTGF/CCN2, FG-3019 and control IgG were gifts from FibroGen, Inc. Fluorescein isothiocyanate-phalloidin was purchased from Molecular Probes (Carlsbad, CA). Recombinant human CTGF was expressed and purified from CTGF baculovirus-infected insect cells and FG-3019, a fully human IgG1x monoclonal antibody that recognizes amino acids 142-157 in domain 2 of human and rodent CTGF, were obtained from FibroGen, Inc.

**CTGF Treatment**

ARPE-19 cells were rendered quiescent by extensively washing the cells with PBS and serum-starving them for 24 hours before stimulating with rhCTGF (25 ng/mL) for the indicated times. Samples were then lysed and subjected to analysis by SDS-PAGE Western immunoblotting.

**Cell Adhesion of ARPE-19**

Plates (96-well) or 8-well slides (Permanox; Nalge Nunc International, Rochester, NY) were coated with FG-3019 (10 \(\mu\)g/mL) for 24 hours. Wells were coated with fibronectin (10 \(\mu\)g/mL) or CTGF (10 \(\mu\)g/mL) for an additional 24 hours and then blocked with 1% BSA (10 \(\mu\)g/mL) for 1 hour at room temperature. Cells were allowed to adhere in the incubator at 37°C 5% CO\(_2\) for up to 3 hours. Adherent cells were then washed with PBS, fixed with 3.7% paraformaldehyde in PBS, permeabilized in 0.01% Triton X-100, followed by staining with either a nucleic acid cell stain (Hoechst 33342; Molecular Probes, Eugene, OR) for the cell count adhesion assay or with FITC-phalloidin (actin cytoskeleton staining) for visualization of cell adhesion and cell spreading. Cells were then visualized and counted (three separate frames) using a microscope with digital vision software (Zeiss Axioplan 2 with AxioVision 4; Carl Zeiss, Inc., Thornwood, NY).

**Aqueous Humor Sampling**

**Patient Recruitment/Ethics.** This study was approved by the Research Ethics Committee of the Mater Misericordiae
FIGURE 1. FG-3019 reduces CTGF induced Erk 1/2 phosphorylation and CTGF-mediated retinal epithelial cell adhesion and cell spreading. (A) ARPE-19 cells were treated with CTGF (25 ng/mL) for 0 to 30 minutes. Whole cell lysates were analyzed by Western blot. Connective tissue growth factor caused a marked increase pErk1/2, and pretreatment with FG-3019 abrogated this effect. Wells were coated overnight with FG-3019 following by coating with either CTGF or fibronectin as a positive control for a further 24 hours. FG-3019 reduced CTGF-mediated adhesion (B) and cell spreading (C) when compared with CTGF alone.
University Hospital and adhered to the tenets of the Declaration of Helsinki. Written informed consent was obtained for all participants.

Sampling. Patients with the following conditions were recruited to the study: PXFG, POAG, and cataract controls. After obtaining informed consent, AqH (50–100 μL per patient) was collected from patients who were undergoing cataract or glaucoma surgery. The removal of AqH was performed as previously described. ⋆ ⋆ ⋆ Pseudoexfoliation and primary open angle glaucoma patients were defined as previously described. ⋆ ⋆ Sixteen cataract patients (73.8 ± 9.7 years), 14 POAG patients (72.3 ± 7.4 years), and 17 PXFG (80.7 ± 7.2 years) were recruited and all were age- and sex-matched (P ≥ 0.05). Two to three AqH samples were pooled per experiment, depending on the original sample volume (50–100 μL per patient).

Cell Exposure to PXFG, POAG, and Cataract AqH Samples in the Presence or Absence of FG-3019

Lamina cribrosa (NLC215, GLC201) and TM cells (NTM160, GTM460) were cultured to confluence and placed in serum-free media (SFM) for 24 hours. Cells were pretreated with FG-3019 (+) or without (−) FG-3019 (10 μg/mL) 1 hour before the cells were exposed to aqueous humor samples. FG-3019 treatment significantly reduced this induced gene expression. Incubation of TM cells with control IgG or FG-3019 (FG) alone did not have any significant effect on gene expression (P > 0.05). In some cases, there were modest increases in gene expression following incubation with aqueous humor from CAT patients. While one increase was statistically significant, in all other cases they were not significant and/or below the levels produced by PXFG and POAG. Results are representative of three independent experiments. (B) Total protein lysates from NTM cells were probed for expression of COL1A1 and αSMA by Western immunoblot. Incubation of cells with all three aqueous humor samples without FG-3019 (−) results in an increase in expression of COL1A1 and αSMA. These increases were blocked by pretreatment with FG-3019 (+). GAPDH was used to ensure equal protein loading.
Figure 3. Induction of ECM production in glaucomatous trabecular meshwork cells by aqueous humor from PXFG and POAG patients is reduced by pretreatment with FG-3019. (A) Following exposure of GTM cells to aqueous humor samples from PXFG and POAG, there was a significant ($P < 0.05$) increase in the expression of profibrotic ECM genes FB, FN, CTGF, COL1A1, and αSMA compared with control cells cultured without aqueous humor. These findings indicate that exposure of TM cells to aqueous humor samples from PXFG and POAG can drive the expression of profibrotic genes. Cells were preincubated with (+) or without (−) FG-3019 (10 μg/mL) 1 hour before the cells were exposed to aqueous humor samples. FG-3019 significantly reduced this gene expression ($P < 0.05$). Incubation of TM cells with control IgG or FG-3019 (FG) alone did not have any significant effect on gene expression ($P > 0.05$). In some cases, there were modest increases in gene expression following incubation with aqueous humor from CAT patients. While one increase was statistically significant, all other cases were not significant and/or below the levels produced by PXFG and POAG. Results are representative of three independent experiments. (B) Total protein lysates from GTM cells were probed for expression of COL1A1 and αSMA by Western immunoblot. Incubation of cells with aqueous humor samples without FG-3019 (−) resulted in a slight increase in expression of COL1A1 and αSMA, which was subsequently reduced by pretreatment with FG-3019 (+). GAPDH is used to ensure equal protein loading.
FIGURE 4. Induction of ECM production in normal and glaucoma lamina cribrosa cells by aqueous humor from PXFG and POAG patients is reduced by pretreatment with FG-3019. (A) Following exposure of NLC cells to aqueous humor samples from PXFG and POAG, there was a significant ($P < 0.05$) increase in the expression of profibrotic ECM genes FB, FN, CTGF, COL1A1, and $\alpha$SMA compared with control cells cultured without aqueous humor. This significant increase was also observed in GLC cells (B). These findings indicate that exposure of LC cells to aqueous humor samples from PXFG and POAG can drive the expression of profibrotic ECM genes. Cells were preincubated with (+) or without (-) FG-3019 (10 μg/mL).
hour before the cells were exposed to aqueous humor samples. FG-3019 reduced profibrotic gene expression in both NLC (A) and GLC (B) cells. Incubation of LC cells with control IgG or FG-3019 (FG) alone did not have any significant effect on gene expression (P > 0.05). In many cases, there were significant increases in gene expression following incubation with aqueous humor from CAT patients (P < 0.05). However, in most cases they were either below the levels produced by PXFG and POAG aqueous humor, especially in the NLC cells. Results are representative of three independent experiments.

(10 µg/mL) for 1 hour. Cells were then incubated with AqH samples obtained from PXFG, POAG, or cataract control patients for 1 hour in SFM and then placed in fresh SFM for an additional 24 hours. Two to three patient samples were used per experiment. Samples were diluted (1 in 10) in 1 mL media per well. FG-3019 (10 µg/mL) or control IgG (10 µg/mL) antibodies alone were also incubated as controls. Results are representative of three independent experiments. Cells exposed to AqH in the presence or absence of FG-3019 were also included in the experiment for protein analysis (NTM 416, GTM 686). Total cellular protein was then examined by SDS-PAGE Western immunoblotting.

**Cell Viability (Crystal Violet Assay) for H₂O₂ Treatment**

Trabecular meshwork (NTM416, GTM473) and LC (NLC176, GLC358) cells were grown to confluence as described above and then placed in SFM for 24 hours. Cells were then treated with H₂O₂ (200–600 µM) for 1 hour and cell viability assessed after 24 hours. In the control cultures, the media was changed at the same time points but H₂O₂ was not added. The viability assay was performed as previously described. Results are representative of three independent experiments.

**Effect of FG-3019 on H₂O₂-Treated TM and LC Cells**

Trabecular meshwork (NTM444, GTM686) and LC (NLC186, GLC428) cells were grown to confluence and placed in SFM for 24 hours. Cells were then pretreated with 10 µg/mL FG-3019 or control IgG (10 µg/mL) for 1 hour. Control cells (antibody) were also included. Cells were then treated with 200 µM H₂O₂ for 1 hour and placed in fresh SFM for an additional 24 hours and fold change in gene expression in the presence or absence of FG-3019 was determined. FG-3019 (10 µg/mL) or control IgG (10 µg/mL) antibodies alone were also incubated with control cells. Results are representative of three independent experiments. Cells (NTM 416, GTM 686) treated with H₂O₂ in the presence or absence of FG-3019 were also included in the experiment for protein analysis. Total cellular protein was then examined by SDS-PAGE Western immunoblotting.

**RNA Isolation, cDNA Conversion, and q-PCR**

Total RNA was extracted and converted to cDNA as previously described. cDNA was assayed in triplicate using a real-time PCR instrument (Rotor-Gene 3000; Corbet Research, Sydney, Australia) and cyanine dye (SYBR Green I; Qiagen, Mannheim, Germany). Primers (Sigma-Aldrich) were designed by a real-time PCR primer design program (Qprimer-depot; http://primerdepot.nci.nih.gov) and were as follows: 18s forward, 5'-gtaacccgtttgaacccatt-3'; 18s reverse, 5'-ggttaccaatgacaacgcct-3'; COL1A1 forward, 5'-gggtaccaatgacaacgcct-3'; COL1A1 reverse, 5'-aatccttgctggccggtgct-3'; fibronectin-1 forward, 5'-aataacacaaggaacctgag-3'; fibronectin-1 reverse, 5'-atccacctggcagaccaaggag-3'; CTGF forward, 5'-tagctggagttgatttggaa-3'; CTGF reverse, 5'-gtttacactggatagtgacgg-3'; fibronectin-1 forward, 5'-tctacagttgttttttggtt-3'; fibronectin-1 reverse, 5'-aatccttgctggccggtgct-3'; CTGF forward, 5'-tagctggagttgatttggaa-3'; CTGF reverse, 5'-gtttacactggatagtgacgg-3'; fibronectin-1 forward, 5'-tctacagttgttttttggtt-3'; fibronectin-1 reverse, 5'-aatccttgctggccggtgct-3'. Predesigned assays (Quantitect Probe Assays; Qiagen) were used for collagen type I alpha1 (COL1A1; QF00530467) and α-SMA (QF00121849). Polymerase chain reaction cycle was 95°C for 10 minutes, 95°C for 30 seconds, 72°C for 30 seconds, 72°C for 5 minutes for 40 cycles. The 2^ΔΔCt method was used to derive a fold difference for gene expression.

**SDS-PAGE Immunoblotting**

Total protein lysates from TM cells were obtained as previously described. Anti-phospho-p44/42 MAP Kinase (Thr202/Tyr204) and anti-p44/42 MAP Kinase, antibodies were obtained from Cell Signaling Technology (Beverly, MA); COL1A1 (sc-28657) was obtained from Santa Cruz Biotechnology (Dallas, TX); anti-α-SMA (ab5694) was obtained from Abcam (Cambridge, UK); and anti-GAPDH (G9545) was obtained from Sigma-Aldrich. Antibodies were incubated in 5% nonfat dry milk overnight. Membranes were then washed in TWEEN-Tris buffered saline and incubated with 1:10,000 dilutions of HRP-linked secondary antibody (anti-rabbit IgG, Cell Signaling Technology). Secondary antibody incubations were carried out at room temperature for 1 hour. Blots were developed using enhanced chemiluminescence reagents (Amersham Biosciences, Buckinghamshire, UK).

**Statistical Analysis**

Data are represented as the mean ± SD and were analyzed using either the Student’s t-test or one-way ANOVA with post hoc Bonferroni testing. P < 0.05 was considered statistically significant and indicated in appropriate figures by an asterisk (*). P < 0.01 is indicated by a double asterisk (**); and P < 0.001 is indicated by a triple asterisk (***)

**Results**

CTGF-Induced Erk1/2 Phosphorylation Is Diminished in Response to Pretreatment With FG-3019

To demonstrate that FG-3019 works through inhibition of CTGF, we assessed its effect on CTGF-dependent phosphorylation of Erk1/2 in ARPE-19 cells. We have previously shown that exogenously added CTGF induced a rapid and transient phosphorylation of Erk1/2 (extracellular signal-regulated kinase 1/2), pERK1/2, and p44/42 MAPK resulting in fibronectin induction and actin cytoskeleton rearrangement. ARPE-19 cells were pretreated with vehicle control or FG-3019 for 1 hour followed by addition of CTGF for 0 to 30 minutes (Fig. 1A). Pretreatment with FG-3019 prevented CTGF-mediated pERK1/2 phosphorylation when compared with control IgG-treated cells and thus demonstrated that FG-3019 elicits its effects through CTGF inhibition.

FG-3019 Abrogates CTGF-Mediated Retinal Epithelial Cell Adhesion and Cell Spreading

Connective tissue growth factor, along with other members of the CCN family of proteins, plays a key role in a number of cellular processes including mitosis, extracellular matrix production, apoptosis and migration, embryogenesis, and adhesion. Connective tissue growth factor mediates its adhesive capacity via binding of integrins and proteoglycans. To
investigate the effect of FG-3019 on the adhesiveness of ECM production. Incubation of cells with FG-3019 (FG) or IgG alone did not cause a significant change in gene expression (Table S2). This increase was significantly diminished by pretreatment with FG-3019, fibrillin-1 (P < 0.01), and ∝-SMA (P < 0.01). There also was an increase in the protein expression of COL1A1 and ∝-SMA in NTM cells following incubation with AqH derived from PXFG, POAG, and cataract patients (Fig. 2B). However, these increases were ameliorated by pretreatment with FG-3019.

Incubation of GTM cells with PXFG or POAG AqH increased the mRNA expression of fibrillin-1 (P < 0.02, P < 0.07), fibronectin-1 (P < 0.03, P < 0.02), CTGF (P < 0.01, P < 0.01), COL1A1 (P < 0.01, P < 0.01), and ∝-SMA (P < 0.03, P < 0.03) compared with untreated control cells (Fig. 3A, Supplementary Table S2). This increase was significantly diminished by pretreatment with FG-3019, fibrillin-1 (P < 0.05, P < 0.08), fibronectin-1 (P < 0.05, P < 0.004), CTGF (P < 0.05, P < 0.01), COL1A1 (P < 0.005, P < 0.01), and ∝-SMA (P < 0.02, P < 0.05). There was a slight increase in protein expression of COL1A1 and ∝-SMA following incubation with AqH (Fig. 3B) from PXFG, POAG, and cataract (CAT) patients and these increases were reduced by pretreatment with FG-3019 (Fig. 3D).

Incubation of NLC cells with AqH from either PXFG or POAG patients significantly increased the gene expression of fibrillin-1 (P < 0.02, P < 0.01), fibronectin-1 (P < 0.03, P < 0.05), CTGF (P < 0.02, P < 0.07), COL1A1 (P < 0.02, P < 0.01), and ∝-SMA (P < 0.01, P < 0.005) compared with untreated control cells (Fig. 4A, Supplementary Table S3). This increase in profibrotic gene expression was diminished by pretreatment with FG-3019, fibrillin-1 (P < 0.04, P < 0.02), fibronectin-1 (P < 0.02, P < 0.03), CTGF (P < 0.04, P < 0.02), COL1A1 (P < 0.02, P < 0.003), and ∝-SMA (P < 0.02, P < 0.001) when compared with AqH alone. Incubation of GLC cells with PXFG or POAG AqH also significantly increased the gene expression of fibrillin-1 (P < 0.02, P < 0.01), fibronectin-1 (P < 0.01, P < 0.01), CTGF (P < 0.01, P < 0.01), COL1A1 (P < 0.01, P < 0.01), and ∝-SMA (P < 0.01, P < 0.01) compared with untreated control cells (Fig. 4B, Supplementary Table S4). This increase was significantly diminished by pretreatment with FG-3019, fibrillin-1 (P < 0.02, P < 0.03), fibronectin-1 (P < 0.02, P < 0.02), CTGF (P < 0.03, P < 0.06), COL1A1 (P < 0.003, P < 0.09), and ∝-SMA (P < 0.01, P < 0.001) indicating that FG-3019 is indeed effective in blocking AqH-mediated ECM production. Incubation of cells with FG-3019 (FG) or IgG alone did not cause a significant change in gene expression (P > 0.1).

**Pretreatment of TM and LC Cells With FG-3019 Blocks H₂O₂-Induced ECM Production**

Using a sublethal dose of H₂O₂ (200 μM; Fig. 5), we investigated whether normal and glaucoma TM and LC cells increased the expression of profibrotic ECM genes.

Treatment of NTM cells with H₂O₂ significantly increased the gene expression of fibrillin-1 (P < 0.008), fibronectin-1 (P < 0.05), CTGF (P < 0.01), COL1A1 (P < 0.01), and ∝-SMA (P < 0.02; Fig. 6A, Supplementary Table S5). Gene expression was significantly diminished by pretreatment with FG-3019, fibrillin-1 (P < 0.002), fibronectin-1 (P < 0.005), CTGF (P < 0.01), COL1A1 (P < 0.02), and ∝-SMA (P < 0.01). Hydrogen peroxide exposure did not significantly increase protein levels of COL1A1 and ∝-SMA. However, pretreatment with FG-3019 blocked expression of these profibrotic ECM proteins (Fig. 6B).

Similar results were observed after H₂O₂ treatment of GTM cells for fibrillin-1 (P < 0.03), fibronectin-1 (P < 0.05), CTGF (P < 0.008), COL1A1 (P < 0.05), and ∝-SMA (P < 0.02; Fig. 7A, Supplementary Table S6). This increase in gene expression was significantly diminished by pretreatment with FG-3019, fibrillin-1 (P < 0.005), fibronectin-1 (P < 0.04), CTGF (P < 0.005), COL1A1 (P < 0.02), and ∝-SMA (P < 0.01). We did not observe...
a significant increase in COL1A1 or α-SMA protein expression following H2O2 treatment. However, protein levels of COL1A1 and α-SMA were reduced in cells pretreated with FG-3019 (Fig. 7B).

Treatment of NLC cells with H2O2 significantly increased the gene expression of fibrillin-1 (P < 0.004), fibronectin-1 (P < 0.01), CTGF (P < 0.02), COL1A1 (P < 0.01), and α-SMA (P < 0.03) compared with untreated control cells (Fig. 8A, Supplementary Table S7). This increase in gene expression was significantly diminished by pretreatment with FG-3019: fibrillin-1 (P < 0.0004), fibronectin-1 (P < 0.008), CTGF (P < 0.01), COL1A1 (P < 0.01), and α-SMA (P < 0.02). Similar results were observed in GLC (Fig. 8B) for fibrillin-1 (P < 0.01), fibronectin-1 (P < 0.05), CTGF (P < 0.01), COL1A1 (P < 0.02), and α-SMA (P < 0.05). This fibrotic response is significantly reduced by pretreatment with FG-3019: fibrillin-1 (P < 0.008), fibronectin-1 (P < 0.02), CTGF (P < 0.001), COL1A1 (P < 0.02), and α-SMA (P < 0.04).

**DISCUSSION**

Elevated IOP is the most common risk factor for developing glaucoma. Current therapies focus solely on lowering IOP and do not address the pathogenic fibrotic processes in the TM and LC or directly protecting the optic nerve axons. This is especially significant in a subset of patients that show disease progression while maintaining normal IOP. We demonstrated that pretreatment with anti-CTGF (FG-3019) reduced the production of fibrotic ECM genes in human TM and LC cells from human donors following exposure to AqH samples from PXFG and POAG patients (Figs. 2–4), or H2O2 treatment (Figs. 6–8). Initially, we demonstrated that FG-3019 inhibits CTGF by effecting associated cellular functions such as induction of Erk1/2 phosphorylation previously described to induce fibronectin and CTGF-mediated adhesion/cell spreading54 (Fig. 1).

We have previously shown that the CTGF levels in AqH of patients with PXFG were significantly higher than in POAG and normal control subjects. Therefore, we examined the fibrotic potential of AqH samples from PXFG and POAG patients using nonglaucomatous cataract samples as controls. Exposure of TM and LC cells to AqH from both PXFG and POAG patients significantly increased expression of profibrotic genes. Other groups have shown that the AqH from glaucoma patients contains elevated levels of markers of oxidative stress, as pro-oxidants have previously been described in the AqH of glaucoma patients.60–62 Elevated levels of TGFβ1 are present in...
the AqH of PXFG patients and TGFβ2 is elevated in POAG AqH.\(^{34,35}\) The presence of both elevated levels of pro-oxidants, CTGF and TGFβ, in the glaucoma aqueous samples may explain the strong induction of fibrotic genes by AqH samples compared with \(\text{H}_2\text{O}_2\) treatment. Pseudoxfoliation glaucoma AqH samples showed the highest levels of CTGF when compared with POAG and cataract control.\(^{36}\) In our study, PXFG AqH samples caused the greatest increase in expression of fibrotic ECM genes in both normal TM and LC cells compared with POAG and cataract samples. The magnitude of responses of normal and glaucomatous TM and LC cells following incubation with AqH. This induction was blocked by pretreatment with FG-3019. In some experiments, we also observed low-level induction of fibrotic gene expression by cataract AqH samples, suggesting that this AqH also contains factors that promote fibrosis in these cells. Normal AqH does contain lower levels of CTGF and TGFβ2. A further explanation is that we did not exclude patients from our study with systemic fibrotic diseases (such as diabetic nephropathy or sarcoidosis), and this may well explain how some cataract aqueous humor samples may indeed function as stimulating agents.

In our study, we exposed cells to \(\text{H}_2\text{O}_2\) in an attempt to mimic the oxidative stress environment associated with the glaucomatous TM and LC tissues. Oxidative stress is thought to be associated/correlated with the pathogenesis of glaucoma.\(^{63}\) Treatment of TM and LC cells with \(\text{H}_2\text{O}_2\) can induce the expression of profibrotic ECM genes. This finding is consistent with other studies that have shown \(\text{H}_2\text{O}_2\)-induced expression of fibronectin-1 in TM cells,\(^{64}\) expression of fibronectin-1, and CTGF in ONH astrocytes.\(^{65}\) Protein levels of COL1A1 and α-SMA are also reduced in cells pretreated with FG-3109. However, in contrast to the changes we saw at the gene expression level, we did not observe a significant increase in protein expression following \(\text{H}_2\text{O}_2\) treatment.

Comparable with TGFβ, CTGF is also upregulated in numerous diseases associated with aberrant ECM deposition, including renal and pulmonary fibrosis.\(^{66,67}\) Importantly and unique to our study is that we demonstrate that pretreatment of TM and LC cells with anti-CTGF significantly reduced the expression of the profibrotic ECM genes induced by \(\text{H}_2\text{O}_2\) in...
normal and glaucomatous TM and LC cells. These data are supported by Junglas et al., 68 who showed CTGF is a critical mediator of the effects of TGFβ2 on ECM synthesis in human TM cells. siRNA knockdown of CTGF inhibited TGFβ2-induced upregulation of fibronectin and CTGF. A study by Wang et al. 48 demonstrated that CTGF and TGFβ cooperate to promote fibrosis and that inhibition of CTGF alone can ameliorate the observed fibrotic phenotype. There appears to be a minimum critical threshold level of CTGF that is required to initiate/maintain fibrosis, and if this threshold is reduced, fibrogenesis is diminished. Furthermore, other studies have shown that CTGF mediates some of the TGFβ effects on ECM production, including collagen and fibronectin. 27,54 This makes CTGF a very attractive therapeutic target for combating the fibrotic process associated with glaucoma pathogenesis. This would provide a novel disease-modifying therapy for the treatment of both the glaucomatous TM and optic nerve head.

In summary, recent studies have described the complex relationship between increased CTGF expression, TGFβ activity, and fibrotic/patent microenvironment in the glaucoma AqH contains sizable quantities of both factors. Importantly, CTGF itself may not be sufficient to promote fibrosis, strengthening the view that CTGF functions as a coordinator of other profibrotic factors such as TGFβ. We believe that the results presented here are supportive of a role for anti-CTGF therapy in combating the pathological fibrotic process associated with glaucoma.

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References


