Mitochondrial Complex I Defect Induces ROS Release and Degeneration in Trabecular Meshwork Cells of POAG Patients: Protection by Antioxidants

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PurPOSE. There is growing evidence that oxidative stress contributes to the progression of primary open-angle glaucoma (POAG), a leading cause of irreversible blindness worldwide. The authors provide evidence that mitochondrial dysfunction is a possible mechanism for the loss of trabecular meshwork (TM) cells in persons with POAG.

Methods. TM from patients with POAG (GTM) and age-matched subjects without disease (NTM) were obtained by standard surgical trabeculectomy. Primary TM cultures were treated with one of the following mitochondrial respiratory chain inhibitors: rotenone (ROT, complex I inhibitor), thionylfluorocetone (TFT, complex II inhibitor), myxothiazol or antimycin A (MYX, AM-complex III inhibitors); mitochondrial permeability transition (MPT) inhibitor cyclosporine A (CSA); and antioxidants vitamin E (Vit E) or N-acetylcysteine (NAC). Mitochondrial function was determined by changes in mitochondrial membrane potential (ΔΨm) and adenosine triphosphate (ATP) production with the fluorescent probes 5,5'-/H11032 and 3,3'-/H9004/H9023-tetracarbocyanine iodide (JC-1) and a luciferin/luciferase-based ATP assay, respectively. Reactive oxygen species (ROS) level, determined by H2DCF-DA, and cell death, measured by lactate dehydrogenase activity and Annexin V-FITC labeling, were also examined.

Results. GTM cells have higher endogenous ROS levels, lower ATP levels, and decreased ΔΨm and they are more sensitive to mitochondrial complex I inhibition than their normal counterparts. ROT induces a further increase in ROS production, the release of cytochrome c, and decreases in ATP level and ΔΨm in GTM cells, eventually leading to apoptosis. Complex II and III inhibition had little effect on the cells. Antioxidants protect against ROT-induced death by inhibiting ROS generation and cytochrome c release.

Conclusions. The authors propose that a mitochondrial complex I defect is associated with the degeneration of TM cells in patients with POAG, and antioxidants and MPT inhibitors can reduce the progression of this condition. (Invest Ophthalmol Vis Sci. 2008;49:1447-1458) DOI:10.1167/iovs.07-1361

Glaucoma, which affects approximately 70 million people, especially in the aging population, is the second leading cause of irreversible blindness worldwide.¹ Primary open-angle glaucoma (POAG) is the most common form of this disease. Blindness caused by POAG is especially prominent in the elderly²–⁴; more than 82% of all blind persons are 50 years of age and older.⁵

POAG is generally associated with elevations in intraocular pressure (IOP) caused by abnormal resistance of aqueous outflow through the trabecular meshwork (TM), a specialized tissue lining the outflow pathway of the eye.⁶ Elevated IOP can lead to progressive neuropathy and ganglion cell death in the neural retina, conditions that often result in irreversible loss of vision.⁶–¹² It has been suggested that age- and disease-related loss of TM cells, followed by substitution with extracellular matrix, contributes to an increased resistance to aqueous outflow and the subsequent increase in IOP found in patients with POAG.¹³–¹⁸ Other possible causes of POAG include mutations of specific genes such as the myocilin (MYOC)¹⁹ and optineurin (OPTN)²⁰ genes, vascular dysregulation,²¹,²² and toxicity and mechanical injury induced by IOP.⁹–¹² One key suspect in disease progression that has been given much attention recently is local oxidative stress.²³ Oxidative free radicals and reactive oxygen species (ROS) are reported to trigger degeneration in the human TM and its endothelial cell components,²⁴ subsequently leading to increases in IOP and glaucoma. There is mounting evidence that in the region of the TM, ROS plays a fundamental role in reducing local antioxidant activities,²⁵,²⁶ reducing outflow, and promoting the activities of superoxide dismutase and glutathione peroxidase²⁶ in glaucomatous eyes. Indeed, oxidative damage to the DNA of TM cells is significantly higher in affected patients than in age-matched control subjects,²⁷ as demonstrated by analysis of 8-hydroxy-20-deoxyguanosine (8-OH-dG), the most common oxidative nucleotide modification.²⁷ Additional studies report a significant correlation among 8-OH-dG levels in the TM, increased IOP, and visual field damage.²⁸ The importance of oxidative damage in POAG has been further substantiated by the findings that glaucoma-affected patients have a significant depletion of total antioxidant potential in the aqueous humor,²⁹ an increase in serum antibodies against glutathione-S-transferase,²⁸ a decrease in plasmatic glutathione levels,²⁰ and an increase in lipid peroxidation products in the plasma³⁰ compared with nonaffected persons. These findings provide a basis for the role of oxidative stress in the pathogenesis of glaucoma and provide new insight.

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into the molecular mechanisms involved in this blinding disease.31,32

The mitochondria are the main source of cellular ROS and adenosine triphosphate (ATP) and are key regulators of mechanisms controlling cell survival and death.33–35 Therefore, the health and activity of the mitochondria are central in the aging process. Khaled et al.36 recently reported a spectrum of mitochondrial abnormalities in patients with POAG. These include a decrease in mitochondrial respiratory activity mtDNA sequence alterations. His data provide some of the first evidence for a possible role of mitochondrial dysfunction in the pathogenesis of glaucoma, supported by the subsequent research.37 Others have also suggested a causal relationship between mitochondrial dysfunction and many of the degenerative abnormalities associated with aging.38–41 This relationship is supported by evidence that mitochondrial abnormalities can reduce the ability of the mitochondria to meet ATP demands.42

Because the mitochondria are the principal sites of ROS generation under physiologic conditions, and because ROS can affect the cellularity of the human TM, we compared mitochondrial function in the TM cells of healthy persons and of those with POAG. The TM cells of patients with POAG exhibited cellular senescence and degeneration compared with those of persons without POAG. There was spontaneous generation of ROS, decreased mitochondrial membrane potential (ΔΨm), decreased ATP production, and loss of cell viability in primary cultures of TM cells of patients with POAG compared with those of control subjects without disease. ROS generation was associated with dysfunction at the level of mitochondrial complex I. We also showed that antioxidants Vit E and NAC and the mitochondrial permeability transition (MPT) inhibitor cyclosporine A (CsA) protected POAG TM cells from cytotoxicity by attenuating ROS production and cytochrome c release from the mitochondria and by inhibiting MPT opening. We hypothesized that the defect in the mitochondrial complex I contributes to progressive loss of the TM cells in patients with POAG by promoting excessive mitochondrial ROS production and by decreasing ΔΨm and ATP synthesis. These changes may also result in accelerated aging in TM cells in patients with POAG.

**Materials, and Methods**

**Materials**

All tissue culture reagents were obtained from Gibco BRL (Gaithersburg, MD). Rotenone (ROT), thenoxytrifluoroacetate (TTFA), antimycin A (AM), myxothiazol (MYX), vitamin E (Vit E), N-acetylcysteine (NAC), cyclosporine A (CsA), aristolochic acid (Ara), and luciferin/ luciferase-based ATP assay kit were purchased from Sigma (St. Louis, MO). 2”,7”-Dichlorodihydrofluorescein diacetate (H2-DCF-DA), 5”,6”-tetrachloro-1”,3”,5”-tetraethylbenzimidazolocarbocyanine iodide (JC-1), Hoechst 33342, a mitochondria-specific dye (MitoTracker Red; Molecular Probes, Eugene, OR), and fluorescent dye (Alexa-Fluor 488) monkey anti-mouse secondary antibody were obtained from Molecular Probes (Carlsbad, CA). Annexin V-FITC apoptosis detection kit was obtained from Bender MedSystems (Burlingame, CA). Mouse anti-cytochrome c monoclonal antibody (mAb) was purchased from Promega (Madison, WI), and the lactate dehydrogenase (LDH) assay kit was purchased from Roche Pharmaceuticals (Nutley, NJ). The following antibodies were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA): mouse anti-fibronectin (FN), rabbit anti-laminin (LN), mouse anti-vimentin, rabbit anti-actin polyclonal antibody, mouse anti-neuron specific enolase (NSE), and mouse anti-factor VIII.

Stock solutions of 10 mM ROT and 100 mM Ara were dissolved in dimethyl sulfoxide. Stock solutions of TTFA (20 mM), AM (30 mM), MYX (1 mM), and CsA (3 mM) were dissolved in 95% ethanol. Stock solutions of NAC (600 mM) and Vit E (200 mM) were dissolved in warm sterile water.

**Patient Information: Clinical Findings in Primary Open-Angle Glaucoma**

All patients were treated in accordance with the Declaration of Helsinki. Before surgery, clinical data were collected on each patient; data included age, sex, use of prostaglandin analogs, number of argon laser trabeculoplasty and other ocular surgical interventions, type and duration of glaucoma, IOP, and visual acuity. Glaucoma diagnosis was based on careful clinical eye examination, including slit lamp, optical coherence tomography, gonioscopy, fundus photography, and visual field. All patients underwent slit lamp examination again the day before surgery. IOP levels in the POAG group exceeded 20 mm Hg at the time of surgery. Visual acuity varied from 0.3 to 1.0. A full ophthalmic history of each donor eye is available at the Eye Bank of Zhongshan Ophthalmic Center. According to the records, no known eye diseases were detected in the control samples used in the present study. For the POAG samples, TM tissue was obtained from patients of similar glaucomatous state after diagnoses by glaucoma specialists. These patients received the prostaglandin analogs latanoprost (0.005%) and travoprost (0.004%) for similar lengths of time. To the authors’ best knowledge, there is no literature reporting that this class of medication can induce changes in the conventional outflow pathway through the trabecular meshwork and its cells. Furthermore, none of the patients from whom the TM samples were obtained previously received steroid medications. The average duration of glaucoma for the patients with POAG was approximately 2 years, and none had a record of systemic disease. Tissue from each patient was used to generate primary cultures of TM cells, as described.

**Tissue Procurement and Cell Culture**

Normal human eyes were obtained from the Zhongshan Ophthalmic Center Eye Bank in Guangzhou, China. The procurement of tissue was approved by the Institutional Review Board Committee at the Sun Yat-sen University at Guangzhou. Normal TM cells were derived from eight human donor eyes, without any known ocular diseases, for corneal transplantation less than 24 hours after death. Donor ages ranged from 20 to 60 years. After written informed consent, TM specimens from eight patients with POAG (15–60 years old) were obtained by standard surgical trabeculectomy for therapeutic purposes less than 1 hour after surgery.

TM tissue of each of the eight controls and of patients with POAG was used to generate independent primary cultures of TM cells. Samples were not pooled at any time in these experiments. Primary cultures were used at passages 2 to 3 for each experiment. Each study was conducted six times, and each trial contained three measurements of each sample. Average measurements from these studies were used to generate the data. Briefly, the human TM was carefully dissected from the anterior segments and the whole corneal layer of human donor eyes. Explants were placed in 24-well culture plates (Corning, Cambridge, MA) containing Dulbecco modified Eagle medium (DMEM) supplemented with 15% fetal bovine serum, 2 mM l-glutamine, penicillin (100 U/mL), and streptomycin (100 μg/mL). Cells from the TM migrated from the explants within approximately 7 days and formed a confluent monolayer between 2 to 5 days. Second- or third-passage cells were used for all the studies described here. NTM and GMT cells were seeded at a density of 1 x 10^5 cells/well using six-well tissue culture plates, and micrographs of the cultures were taken 3 days after seeding, at approximately 80% confluence.

The identity of the TM cells in the cultures was examined using well-characterized antibodies to proteins considered to be TM-specific markers,46–50 including fibronectin (FN), laminin (LN), vimentin, actin, and NSE, and the endothelial cell marker factor VIII.
Treatment with Mitochondrial Complex Inhibitors, Antioxidants, and Mitochondrial Permeability Transition Inhibitor

The optimal dose and duration of the desired mitochondrial inhibitors have been carefully tested on NTM and GTM cells in our laboratory and by others.51,52 In these experiments, the various drugs were used at the following concentrations, as indicated in the literature and from results of prescreening tests we initially performed in our laboratory. To test for mitochondrial functions, 5 μM ROT, 10 μM TTFA, 50 μM AM, or 1 μM MYX was added to the cells. The protective effect of 200 μM Vit E, 10 mM NAC, 3 μM CSA + 50 μM MAA was examined by adding these agents to the cells 30 minutes before the ROT treatment.

To measure ROS, ΔΨm, and cell death and to assess morphologic changes in nontreated cultures and in cultures treated by the various agents, cells were incubated with the indicated agents for 1 hour. For cellular ATP detection, apoptosis, and cytochrome c release, cells were treated with ROT for 0, 4, 8, 12, and 24 hours, respectively.

Measurement of Reactive Oxygen Species

Cellular oxidative stress was determined by the amount of cytoplasmic ROS.53,54 Briefly, suspending treated and untreated cells at a density of 2 × 10⁶ cells/mL were incubated with freshly prepared H₂DCF-DA at 37°C in the dark. H₂DCF-DA penetrates cells and emits green fluorescence on oxidation through reaction with H₂O₂ and, to a certain extent, with nitric oxide. To yield stable and reproducible results, we used 0.4 μM H₂DCF-DA for 30 minutes for flow cytometry and 1 μM for 30 minutes for confocal microscopy.

For flow cytometry, H₂DCF-DA–loaded cells were rinsed twice in PBS and analyzed immediately by flow cytometry at 488 nm excitation and 530 nm emission. Ten thousand cells were routinely collected, and data were expressed as the median fluorescence intensity in arbitrary units from the average of at least three separate experiments.

Measurement of Mitochondrial Membrane Potential

JC-1, a ΔΨm indicator, was used to demonstrate the changes in ΔΨm in TM cells. JC-1 is a lipophilic and cationic dye that permeates plasma and mitochondrial membranes. The dye fluoresces red when it aggregates in the matrix of healthy, high-potential mitochondria, whereas it fluoresces green in cells with low ΔΨm. The JC-1 was freshly diluted in serum-free DMEM to a final concentration of 1 μM/mL and was added to suspending treated and nontreated cells at a density of 2 × 10⁶ cells/mL. After incubation for 20 minutes at 37°C in the dark, all samples were rinsed twice in PBS and analyzed immediately by flow cytometry at 488-nm excitation. Data were collected at 530-nm emission for green fluorescence and 590 nm for red fluorescence. Results are expressed in arbitrary units as median fluorescence intensity.

Measurement of Cellular ATP

ATP levels were determined using a luciferin/luciferase-based ATP assay. Briefly, cells grown in 96-well plates were exposed to 5 μM ROT, 10 μM TTFA, 30 μM AM, and 1 μM MYX in 50 μL culture medium for 24 hours. After removal of the treatments, cell membrane was permeabilized by 50 μL somatic cell ATP-releasing reagent (FLSAR; Sigma-Aldrich Co., St. Louis, MO) and was allowed to react with 50 μL ATP assay mix reagent (FLAA; Sigma-Aldrich Co.) containing luciferin and luciferase. After 10 minutes’ incubation at room temperature, luminescence was measured with a 0.5-second integration time using a luminometer (Orion II; Berthold Detection Systems, Oak Ridge, TN). The cellular ATP level was expressed as the percentage of luminescence intensity of normal human TM cells.

Characterization of TM Cells

Immunolabeling studies were conducted using specific markers as a molecular index to confirm that the primary cultures obtained from healthy persons and those with POAG were indeed TM cells. Expression of the extracellular matrix proteins FN and LN, the cytoskeletal proteins vimentin and actin, NE, and the endothelial cell marker factor VIII was analyzed in the primary cultures. Briefly, TM cells were seeded onto polylysine (10 μg/mL)-coated glass chamber slides at a density of 2000 cells/chamber. After the cultures were rinsed with PBS, the cells were fixed in ice-cold 4% paraformaldehyde for 15 minutes and treated for 4 minutes in 100 mM phosphate buffer, 1 mg/mL bovine serum albumin (BSA), and 0.2% Triton X-100 to permeabilize the cell membranes. After endogenous peroxidase activity was quenched with 3% H₂O₂, the cells were incubated with 0.5% blocking reagent for 30 minutes (TSA Direct; DuPont-NEN, Boston, MA), then immunolabeled with one of the following antibodies at room temperature for 1 hour: mouse monoclonal anti-FN (1:200), rabbit polyclonal anti-LN (1:200), mouse monoclonal antivimentin (1:200), rabbit polyclonal antiactin (1:200), mouse monoclonal anti-NE (1:200), and mouse monoclonal anti-factor VIII (1:200). After incubation with the primary antibody, the cells were rinsed with PBS and incubated for an additional 45 minutes with biotinylated goat anti-mouse IgG (1:300; Vector Laboratories, Burlingame, CA) or anti-rabbit IgG (1:300; Vector Laboratories) where appropriate, followed by avidin-biotin-peroxidase complex for 10 minutes. After a series of washes, the specimens were treated with 3,3′-diaminobenzidine (DAB)/peroxidase reaction (Vector DAB substrate kit; Vector Laboratories), washed in water, treated with hematoxylin counterstain, washed again, and dried at room temperature. The samples were then dehydrated in a graded series of alcohols and coverslipped with 1,3-diyethyl-8-phenylxanthine. The staining pattern for each antibody was visualized using a phase-contrast microscope (DM IRB; Leica, Wetzlar, Germany).

Measurement of Cytochrome c Release in TM Cells

Release of cytochrome c is a putative event of the mitochondria apoptotic pathway after the loss of ΔΨm. Cytochrome c release was measured using flow cytometry, as previously described55,56 with the minor modifications outlined here, and fluorescence images of the cells were collected using confocal microscopy.

For flow cytometric analysis, the treated cells were collected at 0, 4, 8, 12, and 24 hours. Cell suspensions were washed twice in ice-cold PBS (pH 7.4) and resuspended in 1 mL mitochondrial medium (250 mM sucrose, 10 mM KCl, 20 mM HEPES-KOH, pH 7.5, 1 mM EGTA, 1 mM EDTA, 1.5 mM MgCl₂) supplemented with 1% protease inhibitor mixture. Cells were permeabilized for 30 seconds by vortexing with 0.001% digitonin, followed by centrifugation for 5 minutes at 1000g. Cell pellets were resuspended in 4% paraformaldehyde and incubated for 20 minutes at room temperature. After two washes with PBS (pH 7.4), the fixed cells were incubated in labeling medium containing 2% FBS, 0.2% sodium azide, and 0.5% Triton X-100 in PBS for 15 minutes.

The suspension was centrifugated at 3000g for 5 minutes before incubation with 1 μg/mL mouse monoclonal anti-cytochrome c diluted in 200 μL labeling medium at 4°C for 1 hour. After treatment with the primary antibody, cells were washed twice in the labeling medium and then incubated in 5 μg/mL goat anti-mouse fluorescent dye (AlexaFluor 488; Molecular Probes)-conjugated secondary antibody diluted in 200 μL labeling medium at 4°C for an additional hour. Cells were washed twice, resuspended in 200 μL PBS, and immediately analyzed by flow cytometry.

For confocal microscopy, cells were seeded onto polylysine (10 μg/mL)-coated glass chamber slides at a density of 2000 cells/chamber. After they were washed with PBS, cells were fixed in ice-cold 4% paraformaldehyde for 15 minutes and permeabilized for 4 minutes in 100 mM phosphate buffer, 1 mg/mL bovine serum albumin, and 0.2% Triton X-100. After the endogenous peroxidase activity was quenched with 3% H₂O₂, the cells were incubated with 0.5% blocking reagent for 30 minutes (TSA Direct; DuPont-NEN). The cells were immunolabeled with mouse monoclonal anti-cytochrome c (1:1000 dilution) at room temperature for 1 hour. Normal mouse IgG was used instead of anti-cytochrome c in some experiments to serve as negative controls.
After incubation with the primary antibody, the cells were washed and incubated for 45 minutes with horseradish peroxidase-conjugated goat anti-mouse IgG (1:50), followed by FITC-tyramide solution (1:50) for 10 minutes. Cells were then washed and mounted in fluorescence mounting medium. The staining pattern was visualized with the use of a confocal microscope (100M; Carl Zeiss Jena GmbH, Jena, Germany).

**Morphologic Analysis of TM Cultures**

NTM and GTM cells were seeded at a density of $1 \times 10^5$ cells/well of a six-well plate, and micrographs of all cultures were taken 3 days after seeding (80% confluence) to examine morphologic changes between the two cultures using light and confocal microscopy. Some cells were stained with $1 \mu g/mL$ Hoechst 33342 to illustrate the nuclei, and some of the cells were labeled with 50 nM a mitochondria-specific dye (MitoTracker Red; Molecular Probes) to show the mitochondria.

**Measurement of Cell Death**

Cell death in the cultures was evaluated by measuring LDH activity in the conditioned medium using a colorimetric assay. Briefly, cells were grown in a 96-well plate at a density of $1 \times 10^5$ cells/well and were exposed to $5 \mu M$ ROT, $10 \mu M$ TFFA, $30 \mu M$ AM, or $1 \mu M$ MYX in 50 $\mu L$ culture medium for 1 hour. Then 50 $\mu L$ culture supernatant of each sample was transferred to a fresh 96-well plate and was allowed to react with 50 $\mu L$ reaction mixture solution at room temperature for 30 minutes. After termination of the reaction by the addition of a stop solution, absorbance at 490 nm was measured on a microplate reader (Benchmark Microplate Reader; Bio-Rad, Hercules, CA). The amount of cell death was directly proportional to the LDH activity and was estimated as a percentage according to the internal no treatment control and total cell death control.

Apoptosis was also examined using reagents from an Annexin V-FITC apoptosis detection kit according to the manufacturer's protocol. Briefly, cells were grown in a six-well microtiter plate at a density of $1 \times 10^5$ cells/well. After treatment with $5 \mu M$ ROT for 24 hours, cells were harvested and resuspended in 1$X$ binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl$_2$, 1:4 dilution) and then centrifuged at 800 $g$ for 2 minutes. Then the cells were resuspended in 100 $\mu L$ binding buffer containing 2.5 $\mu L$ Annexin V-FITC and were incubated in the dark at room temperature for 15 minutes; this was followed by the addition of 10 $\mu L$ of 20 $\mu g/mL$ PI. Cells were analyzed by flow cytometry immediately afterward.

**Statistical Analysis**

All assays were performed using at least six separate experiments in triplicate, and data were expressed as mean $\pm$ SE. One-way ANOVA was performed, and statistical significance was set at $P < 0.05$.

**RESULTS**

Several reports indicate that pathologic conditions in the TM of patients with POAG contribute to the progression of this disease. Many have suggested that oxidative stress plays an important role in promoting increased IOP in these patients. Here we studied the levels of endogenous ROS generation, ATP production, and cytochrome $c$ release by TM cells obtained from patients with POAG (GTM) and age-oxidation-sensitive fluorescence dye $H_2$DCF-DA show that there are more labeled cells and greater labeling intensity in GTM than in NTM cells. Scale bar, 30 $\mu m$. (C) Distribution of $H_2$DCF-DA fluorescence intensity in TM cell cultures using flow cytometry. GTM cultures have stronger $H_2$DCF-DA fluorescence intensity than NTM cultures. (D) Relative amounts of total $H_2$DCF-DA fluorescence intensity in TM cultures. Data are expressed as fold changes in fluorescence levels of GTM to NTM. Results are expressed as the mean $\pm$ SE of six separate experiments performed in triplicate.
matched controls (NTM) and the sensitivity of these cells to various mitochondrial inhibitors and antioxidants.

TM Cell Characterization

In Figure 1A, we confirmed that the primary cultures obtained from healthy subjects and patients with POAG were indeed TM cells because they were immunopositive for TM-specific molecular markers, extracellular matrix molecules FN and LN, cytoskeletal molecules vimentin and actin, and NSE. The cultures were also immunonegative for the endothelial cell marker factor VIII, as has been reported by others.46–50

POAG TM Cells Have Higher Endogenous ROS Levels than Nondiseased TM Cells

In Figure 1B, we showed that cultured GTM and NTM cells have different morphologic appearances when grown as monolayer cultures. In the phase-contrast light micrographs, GTM cells appear much larger and are more irregularly shaped than the nondiseased, age-matched NTM cells. We also showed that the level of endogenous ROS, indicated by H$_2$DCF-DA oxidation in the TM cells, was significantly higher in GTM than in NTM cells by confocal microscopy (Fig. 1B) and flow cytometry studies (Figs. 1C, 1D; $P < 0.05$).

Mitochondrial Complex I Inhibitor Increases ROS Production in GTM but Not NTM Cells

We next examined whether the source of ROS production in the cultured TM cells was generated by the mitochondria of the cells. To do this, we treated the cultured TM cells with various mitochondrial respiratory chain inhibitors to determine which group of cells was more sensitive to them and to determine whether the inhibitors were able to alter ROS production by the cells. In Figure 2, we present evidence that NTM cells had the least amount of ROS production and that treatment with the mitochondrial respiratory chain inhibitor had no significant effects in the ROS level of the NTM cells. On the contrary, mitochondrial complex I inhibitor ROT increased ROS production in GTM cells by 13-fold, whereas inhibitors for complexes II (TTFA) and III (AM and MYX) had no effect. These results were confirmed by confocal microscopy (Fig. 2A) and flow cytometry (Fig. 2B). Given that the mitochondrial complex I defect has been shown to cause increased ROS release from the mitochondria in several cell types,57,58 our data suggest that there is a defect in the mitochondrial complex I in GTM cells. Treatment with other inhibitors, including allopurinol, an inhibitor of xanthine oxidase (another intracellular generator of ROS), had no effect on the GTM cells (data not shown), further confirming that the source of ROS production in the GTM cells was the mitochondria. Although it is difficult to rule out ROS contributions from other cellular systems that produce intracellular oxidants (e.g., nitric oxide synthase and cytochrome P450 enzymes), the studies described strongly suggest that the mitochondria are the most likely principal source of ROS in GTM cells.

ROT Increases ROS Production in GTM Cells in a Dose- and Time-Dependent Manner

The studies in Figure 3 were carried out to determine the concentration of ROT that could induce maximum ROS production by the cultured GTM cells and the time frame in which it does so. As analyzed by flow cytometry, ROT induces ROS production in both a dose- and a time-dependent manner in GTM cells. Treatment with ROT at 0 to 10 $\mu$M for 1 hour caused a linear rise in ROS level in GTM (Fig. 3A). ROT (5 $\mu$M) induced ROS generation, with the maximal effect seen at 60 minutes; longer ROT treatment did not further increase ROS generation in GTM (Fig. 3B). In NTM cells, an elevation in ROS level was observed only under a high concentration (more than

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Mitochondrial complex I inhibitor increases ROS production in GTM but not NTM cells. NTM and GTM cells were treated with the specific inhibitors for the mitochondrial respiratory chain components, the complex I inhibitor ROT (5 $\mu$M), the complex II inhibitor TTFA (10 $\mu$M), and the complex III inhibitors AM (30 $\mu$M) and MYX (1 $\mu$M) for 1 hour. (A) H$_2$DCF-DA fluorescence labeling, using confocal microscopy, shows the increased fluorescence intensity of ROS production in GTM cells after treatment with ROT. TTFA, AM, and MYX did not significantly increase ROS generation in GTM cells. Data are expressed as a fold changes in fluorescence levels of GTM to NTM. Results are expressed as the mean $\pm$ SE of six separate experiments performed in triplicate. *Significant differences from untreated GTM at $P < 0.05$.}
5 μM) of ROT treatment; less than 5 μM ROT could not induce ROS generation in these cells (Fig. 3A).

Figure 3. ROT increases ROS production in GTM in a dose- and time-dependent manner. NTM and GTM cells were loaded with H2-DCF-DA, and ROS level was measured by flow cytometry. Data are expressed as a fold change in ROS production of GTM to NTM. ROS level in NTM cells at time 0 and ROT 0 μM is arbitrarily set at 1. (A) Treatment with ROT for 60 minutes at a concentration range of 0 μM to 10 μM causes a linear rise in ROS level in GTM cells but has little effect on NTM cells. (B) 5 μM ROT induces ROS production in GTM cells as early as 15 minutes after treatment. ROS level continues to rise and reaches a maximum at 60 minutes. Results shown are the mean ± SE of six separate experiments performed in triplicate.

GTM Cells Have Lower Endogenous ATP Levels and ΔΨm than NTM Cells, and ROT Can Further Decrease ATP Production and ΔΨm in GTM Cells

ATP depletion and mitochondrial depolarization (decrease in ΔΨm) are 2 major events in mitochondrial dysfunction. Figure 4A showed that there was a decrease in endogenous ATP level in GTM compared with NTM cells (P < 0.05). After 24-hour treatment with ROT (5 μM), there was an additional significant decline in ATP level by 33% in the GTM but no significant effect on NTM. In addition, GTM had a lower endogenous ΔΨm than NTM (P < 0.05). One-hour exposure of cells to 5 μM ROT led to an additional decline in ΔΨm by 7.89-fold in the GTM cells but no significant effect on NTM cells (Fig. 4B). These two experiments indicated mitochondrial dysfunction in the GTM cultures.

Figure 4. ROT decreases ATP levels and mitochondrial membrane potential in GTM cells. (A) Luciferin/luciferase-based ATP assay indicates that the ATP level in GTM is 30% lower than in NTM cells. 5 μM ROT treatment for 24 hours leads to an additional decline in GTM cells levels of ATP by 33%. The ATP level in control NTM is arbitrarily defined as 100%. Results are expressed as a mean percentage of the ATP levels of the control ± SE of six separate experiments performed in triplicate. *Significant differences from NTM at P < 0.05. (B) ΔΨm was examined using flow cytometry with the fluorescence indicator JC-1. The ΔΨm is 7.29-fold (± 0.90) lower in GTM than in NTM cells. 5 μM ROT treatment for 1 hour leads to an additional decline in ΔΨm by 7.89-fold (± 1.93) in the GTM cells (from 7.29-fold lower to 15.18-fold lower after ROT treatment in GTM compared with NTM). Results are expressed as mean fold change in fluorescence levels of GTM to NTM samples ± SE of six separate experiments performed in triplicate. *Significant differences from NTM at P < 0.05.

ROT Induces Cytochrome c Release from the Mitochondria in GTM Cells

Because it is well known that excessive ROS, decreased ATP, and ΔΨm can induce apoptotic death,33–35 we next examined the release of mitochondrial cytochrome c, an important signaling molecule in apoptosis, in the diseased and nondiseased TM cultures after ROT treatment.

In Figure 5A, GTM and NTM cells showed an exact overlap of anti-cytochrome c and mitochondria fluorescence in confocal micrographs, indicating colocalization of cytochrome c and mito-
There was no release of cytochrome c from the mitochondria in GTM or NTM cells before ROT treatment. After treatment with 5 μM ROT for 24 hours, cytochrome c was observed in the cytoplasm of GTM cells that was not coincident with the mitochondria labeling, indicating that ROT selectively induced the release of cytochrome c from the mitochondria in GTM cells but not in NTM cells. In Figures 5B and 5C, we quantified the ROT-induced cytochrome c release by flow cytometry in a time window of 0 to 24 hours in GTM cells. Treatment with 5 μM ROT caused a time-dependent release of cytochrome c in GTM cells, which was significantly different from that of untreated controls at 12 and 24 hours (P < 0.05).

**ROT Induces GTM Cell Death**

Morphologically, NTM cells maintained a fairly healthy appearance after treatment with 5 μM ROT for 1 hour or 24 hours, whereas a significant number of GTM cells had detached from the culture dish; those that remained looked unhealthy and exhibited cell shrinking and shedding, characteristic features of cell death (Fig. 6A).

In addition, treatment with 5 μM ROT for 24 hours diminished the punctate appearance of the mitochondria in GTM cells, as shown by mitochondria-specific dye (MitoTracker Red; Molecular Probes) staining, whereas there was still punctate staining of the mitochondria of NTM cells (Fig. 6B). Nuclei of the GTM cells showed a distorted appearance compared with the well-preserved nuclei in NTM cells after 24 hours of ROT treatment, as illustrated by Hoechst 33342 staining (Fig. 6B). However, no significant morphologic changes were observed in NTM and GTM cells after TTFA, MYX, or AM treatment (data not shown).

We next used LDH assay to quantify the amount of cell death induced by ROT in the cultures. Incubation with ROT for 1 hour significantly increased LDH release from GTM cells by 4.01-fold (± 0.34) compared with only 1.20-fold (± 0.14) from NTM cells (Fig. 6C). To determine whether cell death was the result of apoptosis, we used Annexin V-FITC staining, an indicator of apoptosis. Here we provide evidence that after 24 hours, treatment with ROT significantly induced a 5.53-fold (± 0.95) increase in apoptosis in GTM cells compared with only a 1.35-fold (± 0.34) increase in NTM cells (Fig. 6D). Taken together, the data suggested that ROT induced apoptotic cell death in GTM cells while it only mildly affected NTM cells.

**Antioxidants and MPT Inhibitor Reduce ROS Generation, Increase Mitochondrial Membrane Potential, and Decrease Cytochrome c Release in GTM Cells**

As indicated in Figure 7A, we showed that antioxidants Vit E and NAC reduced endogenous ROS levels in GTM cells (Fig. 7A). Pretreatment with Vit E or NAC for 30 minutes also effectively reduced ROT-induced ROS production in GTM cells but had no significant effect on NTM cells (Fig. 7A).

Given that mitochondrial depolarization and cytochrome c release are consequences of MPT opening, the protective effect of the MPT inhibitor CsA+ArA was also examined. Pretreatment analysis shows that GTM and NTM cells have exact overlapping cytochrome c staining (green) and mitochondria staining (red), indicating colocalization of cytochrome c and mitochondria. After treatment with 5 μM ROT for 24 hours, some cytochrome c is observed in the cytoplasm of GTM cells, indicating ROT induces the release of cytochrome c from the mitochondria in GTM cells. Scale bar, 30 μm. (B, C) Flow cytometric analysis shows that treatment with 5 μM ROT for various time points (0–24 hours) causes a time-dependent release of cytochrome c in GTM cells at 12 and 24 hours.
with the MPT inhibitor 3 μM CsA+50 μM ArA for 30 minutes prevented the ROT-induced loss in ΔΨm in GTM cells, as shown by flow cytometry (Figs. 7B, 7C). Pretreatment with Vit E, NAC, or CsA+ArA for 30 minutes could also suppress ROT-induced cytochrome c release from the mitochondria of these cells, as illustrated by confocal microscopy (Fig. 7D). These findings implied that ROT-mediated ROS production and MPT opening precede cytochrome c release, a hallmark event of apoptosis.

Antioxidants and MPT Inhibitor Prevent ROT-Induced GTM Cell Death

Finally, we showed that pretreatment of GTM and NTM cultures with the antioxidant Vit E and NAC or the MPT inhibitor CsA+ArA could significantly reduce LDH release from 4.01-fold (± 0.34) to 1.95-fold (± 0.16), 1.75-fold (± 0.08), and 1.58-fold (± 0.17) in ROT-treated GTM cells (Fig. 8A) and could

FIGURE 6. ROT induces GTM cell death. (A) Phase-contrast light micrographs showing a general unhealthy, degenerative appearance of GTM cells after treatment with 5 μM ROT for 1 hour and 24 hours. Fewer cells are seen in these cultures, in part because of cell death and in part because of cell detachment. NTM cells maintain a relatively healthy appearance even after ROT treatment. Results indicate that GTM cells are more sensitive to ROT than NTM cells. Scale bar, 30 μm. (B) Loss of mitochondria (punctate appearance) is more obvious in GTM cells after treatment with 5 μM ROT for 24 hours. Although there is fairly strong staining with the mitochondrial marker, the pattern is more diffuse than with the punctate appearance of the mitochondria in the control NTM cells before and after ROT treatment. In addition, after 24 hours of treatment with ROT, the nuclei of the GTM cells are distorted, as illustrated by Hoechst 33342 staining. Scale bar, 30 μm. (C) ROT induces 4.01-fold (± 0.34) more LDH release from GTM cells but only 1.20-fold (± 0.14) from NTM cells after 1-hour treatment, indicating that GTM cells are more susceptible to death with ROT treatment. Data are expressed as a fold change in LDH release of GTM to NTM. Results are expressed as the mean ± SE of six separate experiments performed in triplicate. *Significant differences from NTM at P < 0.05. (D) Annexin V-FITC staining shows that there is 5.53-fold (± 0.95) more apoptosis in GTM cells compared with only 1.35-fold (± 0.34) more apoptosis in NTM cells after treatment with 5 μM ROT for 24 hours. Data are expressed as a fold change in Annexin-V labeling of GTM to NTM. Results are expressed as the mean ± SE of six separate experiments performed in triplicate. *Significant differences from NTM at P < 0.05.
reduce apoptosis from 5.53-fold (± 0.95) to 2.76-fold (± 0.56), 2.78-fold (± 0.51), and 2.60-fold (± 0.76) compared with the no-treatment cultures (Fig. 8B).

**DISCUSSION**

Loss of cells in the TM is reported to be a clinical feature in patients with POAG. However, the exact molecular mechanism causing TM cell loss in patients with POAG is still obscure. Here we present evidence that ROS production is higher in the TM cells of patients with POAG than in those obtained from age-matched controls without POAG, supporting the previous findings of oxidative damage in TM cells of patients with POAG. Most important, we are the first to describe a decrease in the activity of the mitochondrial respiratory chain complex I in the TM cells from patients with POAG and increased mitochondrial ROS production, decreased ΔΨm and ATP synthesis, increased cytochrome c release, and increased sensitivity of the POAG TM cells to rotenone-induced apoptosis. These findings, taken together, support our hypoth-
mean release of GTM to no treatment NTM. Results are expressed as the fold changes in LDH activities of GTM cells and apoptosis (B) from 4.01-fold (\(\pm 0.34\)) to 1.95-fold (\(\pm 0.16\)), 1.75-fold (\(\pm 0.08\)), and 1.58-fold (\(\pm 0.17\)) from ROT-treated GTM cells and apoptosis (B) from 5.53-fold (\(\pm 0.95\)) to 2.76-fold (\(\pm 0.56\)), 2.78-fold (\(\pm 0.51\)), and 2.60-fold (\(\pm 0.76\)) above the levels of untreated GTM cells. There was a little effect on NTM cells after treatment with these agents. Data are expressed as fold changes in LDH release of GTM to no treatment NTM. Results are expressed as the mean \(\pm\) SE of six experiments performed in triplicate. *Significant differences from GTM+ROT at \(P < 0.05\).

The release of cytochrome \(c\) into the cytoplasm of cells requires MPT opening. \(^{51,62}\) In these studies, we also show that CsA+ArA, the MPT inhibitor, significantly reduces endogenous and ROT-reduced mitochondrial depolarization, cytosolic translocation of cytochrome \(c\) and subsequent apoptosis of TM cells in patients with POAG. Similar effects were observed after removal of the ROS using antioxidants such as Vit E and NAC. Antioxidants have been proposed as likely candidates to reduce ROS production and as complementary agents in the treatment of glaucoma. \(^{63-66}\) Our findings strengthen this proposal and are of clinical importance in the development of targeted glaucoma treatments that would lessen the mitochondrial complex I defect-mediated oxidative damage in TM cells.

In conclusion, we present strong evidence that mitochondrial dysfunction is a possible mechanism for the loss of TM cells in patients with POAG. Evidence also suggests that oxidative stress-induced DNA damage in the TM region is the root of IOP elevations and visual field defects in patients with POAG. \(^{33}\) Furthermore, the oxidative DNA damage of human TM cells was shown to be fivefold higher in patients with glaucoma than in controls; such an increase is independent of the age of the patient or the duration of the disease. \(^{27}\) Our study confirms that endogenous ROS levels are higher in the TM of patients with POAG than in age-matched persons without POAG, indicating that increased oxidative stress is associated with the pathogenesis of POAG. There is a general consensus that cumulative oxidative damage is responsible for general aging. \(^{33-35,39}\) Although many patients with POAG are young adults, the higher ROS level in the TM cells could possibly accelerate the aging process in this region, which may, in part, contribute to the progression of POAG.

Defects in complex I of the mammalian mitochondrial respiratory chain are known to be involved in the increased production of ROS and are linked to several degenerative disorders. \(^{59,60}\) To study whether the increase in ROS production in the TM cells of patients with POAG is a result of mitochondrial complex I defects, we used several inhibitors of mitochondrial function, including ROT (complex I inhibitor), TTFA (complex II inhibitor), and AM and MYX (complex III inhibitors). Our data show that ROT triggers a rapid increase in ROS production over the levels already present in the TM cells obtained from patients with POAG, with no significant changes in the control nondiseased TM cells. Of importance is the finding that there is no significant alteration in ROS production in POAG TM cells after treatment with the inhibitors of complexes II and III, indicating a direct link between mitochondrial complex I defects and elevated ROS level in the TM cells of patients with POAG.

Because of the role of mitochondria as the cell's major energy source, mitochondrial dysfunction underlies key events leading to apoptosis. \(^{33-35}\) Some mitochondrial-specific actions leading to apoptosis include loss of \(\Delta\Psi_m\), induction of MPT opening, and cytosolic translocation of apoptogenic factors such as cytochrome \(c\). \(^{51,62}\) Here we found that the TM cells of patients with POAG have lower levels of ATP and \(\Delta\Psi_m\) than nondiseased TM cells. Treatment with ROT not only causes further ATP depletion and mitochondrial depolarization, it also induces time-dependent mitochondrial cytochrome \(c\) release into the cytoplasm of POAG TM cells, whereas nondiseased TM cells are relatively unaffected by ROT treatment. The higher sensitivity of patients with POAG TM cells to mitochondrial complex I inhibition suggests an intrinsic mitochondrial complex I defect in these cells.

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In conclusion, we present strong evidence that mitochondrial complex I defects are the major source of oxidative stress in TM cells of patients with POAG. New insight into the protective effects of antioxidants and MPT inhibitors for the health of TM cells suggest that there are POAG treatment possibilities worthy of further investigation.
References


