PurPOSE. Recent studies have revealed an accumulation of senescent cells in the outflow pathways in primary open-angle glaucoma (POAG). Transforming growth factor (TGF)-β2 is thought to be involved in the pathologic changes of the trabecular meshwork (TM) of POAG eyes. The goal of this study was to determine whether TGF-β2 triggers senescence-associated changes in human TM cells in vitro.

METHODS. Cultured human TM cells were exposed to 1.0 ng/mL TGF-β2 for 12, 24, and 48 hours. Senescence-associated β-galactosidase (SA-β-Gal) activity was investigated by histochemical staining. Lipid peroxidation was assessed after TGF-β2 treatment. Levels of apolipoprotein J (Apo J), SM22, and osteonectin (SPARC) mRNA were determined by real-time PCR analysis. Furthermore, the effects of antioxidants on these TGF-β2-mediated changes were tested. Induction of senescence-related signal transduction proteins (p16, p21, and pRb) was examined by real-time PCR and Western blot analysis.

RESULTS. TGF-β2 increased SA-β-Gal activity, lipid peroxidation, and the mRNA expressions of Apo J, SM22, and SPARC. These TGF-β2-induced changes were attenuated by antioxidants. TGF-β2 increased p16 mRNA and protein expression, which was paralleled by a downregulation of pRb protein. There was no effect on p21 mRNA and protein expression after exposure to TGF-β2.

CONCLUSIONS. TGF-β2 induces senescence-associated TM changes and activates the senescence-related p16-pRb signal transduction pathway in vitro. Thus, minimizing TGF-β2 levels may help to prevent the ageing process in the TM as seen in POAG. (Invest Ophthalmol Vis Sci. 2010;51:5718–5723) DOI: 10.1167/iovs.10-5679

Primary open-angle glaucoma (POAG) is one of the leading causes of blindness in the world. This optic neuropathy is characterized by elevated intraocular pressure that may be attributable to an increased resistance in the aqueous humor outflow pathways.1,2 Histologic studies have demonstrated that POAG is associated with pathologic changes in the trabecular meshwork (TM).1–3 Recent investigations have revealed an accumulation of senescent cells in the outflow pathways of glaucomatous eyes compared with age-matched control eyes.4 Cellular senescence is characterized by an increased expression of the senescence-associated enzyme β galactosidase (SA-β-Gal), the stress protein apolipoprotein J (Apo J), and the senescence-related biomarkers SM22 and osteonectin (SPARC).5,6 An overexpression of SM22 and SPARC was initially described in senescent human diploid fibroblasts after exposure to oxidative stress.7 SM22 is an actin-binding protein involved in senescence-associated morphologic changes.7,8 SPARC has been shown to be expressed by TM cells and is prominently distributed in the juxtacanalicular region.9 Previously, it has been reported that an increased expression of extracellular matrix (ECM) genes including SPARC is characteristic of oxidative stress-induced senescence of the TM, thus leading to TM malfunction and TM cell loss.10 Therefore, increased amounts of SPARC may be involved in intraocular pressure dysregulation and the pathophysiology of POAG.10

There is a broad range of evidence suggesting that the process of cellular senescence is mediated by specific signal transduction proteins (p16 and p21) leading to the hypophosphorylation of the retinoblastoma (Rb) protein.11–13 From a variety of studies it is known that the exposure to stress factors can trigger and accelerate the development of stress-induced premature senescence (SIPS).6,12 One of the most important factors leading to SIPS is an increased production of reactive oxygen species (ROS) and thus oxidative stress. Another factor that can also generate ROS and promote SIPS is transforming growth factor (TGF)β1–4–17 Increased levels of TGF-β2 have also been detected in the aqueous humor of POAG patients.18,19 The goal of this study was to analyze the role of TGF-β2 in the induction of cellular senescence in cultured human TM cells. First, we wanted to examine whether TGF-β2 can increase lipid peroxidation and thus generate ROS in human TM cells. Furthermore, the influence of antioxidants on the TGF-β2-mediated senescent TM changes was investigated. Finally, the effects of TGF-β2 on the expression of the senescence-associated signal transduction proteins p16, p21, and pRb were examined.

MATERIALS AND METHODS

Cell Culture

Primary cell cultures of human TM were obtained from the eye bank of the Ludwig-Maximilians-University, Munich, Germany. Monolayer cultures were established from eyes of five human donors (25–36 years old, obtained 5–8 hours after death) without any history of eye disease. Methods of securing human tissue were humane, included proper consent and approvals, complied with the Declaration of Helsinki, and were approved by the local ethics committee. TM cells were prepared, grown, and classified as described elsewhere.20–25 The effects of TGF-β2 were investigated by incubating cells of passages 3 to 5 that had been confluent for at least 7 days in serum-free Ham’s F-10 medium (Gibco-Life Science Technology, Karlsruhe, Germany) for 24 hours at 37°C and 5% CO2. Thereafter, the medium was replaced by fresh serum-free Ham’s F-10 medium, and the cells were exposed to 1.0 ng/mL active TGF-β2 (R&D Systems, Wiesbaden, Germany) for 12, 24, and 48 hours. In control cultures, the medium was changed at the same time points, but no TGF-β2 was added. The effects of the antioxidants were determined by pretreating the TM cells with ascor-
bic acid (Sigma-Aldrich, Deisenhofen, Germany) and coenzyme Q10 (Sigma-Aldrich) at a concentration of 10 μg/mL for 48 hours. Afterward, the cells were washed twice with phosphate-buffered saline (PBS) and then exposed to 1.0 ng/mL TGF-β2 for 24 hours. Ascorbic acid was dissolved in Ham’s F-10 medium. Coenzyme Q10 was dissolved in DMSO as a stock solution of 10 mg/mL, which was dissolved further in Ham’s F-10 medium containing 0.5% FCS to yield the desired concentration.

**SA-β-Gal Activity**

The proportion of TM cells positive for SA-β-Gal activity was determined, as described by Dimri et al.24 Briefly, treated TM cells were washed twice with PBS and fixed with 2% formaldehyde and 0.2% glutaraldehyde in PBS at pH 6.0 at room temperature (RT) for 4 minutes. Cells were then washed twice with PBS and incubated under light protection for 8 hours at 37°C with fresh SA-β-Gal staining solution (1 mg/mL; 5-bromo-4-chloro-3-indoyl-p-galactopyranoside [X-Gal], 40 mM citric acid/sodium phosphate [pH 6.0], 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM MgCl2 diluted in PBS). The cells were then examined for the development of blue color and photographed at low magnification (200×) with a light microscope. All experiments were performed at least in triplicate in TM cultures from three donors.

**Assessment of Lipid Peroxidation**

Oxidative stress can be assessed by markers of lipid peroxidation. A sensitive and specific assay for lipid peroxidation is based on metabolic incorporation of the fluorescent oxidation-sensitive fatty acid, cis-parinaric acid (PNA), a natural 18-carbon fatty acid with four conjugated double bonds, into membrane phospholipids of cells.25,26 Oxidation of PNA results in disruption of the conjugated double-bond system that cannot be re-synthesized in mammalian cells. Therefore, lipid peroxidation was estimated by measuring the loss of PNA fluorescence. Briefly, treated cells were incubated with 10 μM PNA (Molecular Probes-Invitrogen, Paisley, UK) at 37°C for 30 minutes in the dark. The medium was then removed and the cells washed three times with PBS. Afterward, the cells were scraped into 2 mL PBS with a rubber policeman. The suspension was then added to a fluorescence cuvette and measured at 512-nm excitation and 455-nm emission. A blank (unlabeled cells) was measured and subtracted from all readings. All experiments were performed at least in triplicate in TM cultures from three donors.

**RNA Isolation and Real-Time PCR**

Total RNA was isolated from 10-mm Petri dishes by the guanidinium thiocyanate-phenol-chloroform extraction method (Stratagene, Heidelberg, Germany). The structural integrity of the RNA samples was confirmed by electrophoresis in 1% Tris-acetate-EDTA (TAE)-agarose gels. Yield and purity were determined photometrically. After RNA isolation, mRNA was transcribed to cDNA via reverse transcriptase. This cDNA was then used for specific real-time PCR. Quantification of human apolipoprotein J (Apo J), SM22, SPARC, p16, p21, and Rb mRNA was performed with specific primers (Table 1) in 40 cycles on a thermal cycler (LightCycler System; Roche Diagnostics, Mannheim, Germany). Primers and probes were generated with proprietary software (ProbeFinder, ver. 2.04; Roche Diagnostics). All primers were extended over exon-intron boundaries. The standard curve was obtained from probes of three different untreated human TM cell cultures. Differences in the amount of total RNA added to each reaction were normalized by simultaneously processing 18S rRNA (Table 1) in the same sample as an internal control. The levels of Apo J, SM22, SPARC, p16, p21, and Rb mRNA were determined as the relative ratio (RR), which was calculated by dividing the level of Apo J, SM22, SPARC, p16, p21, and Rb mRNA by the level of the 18S rRNA housekeeping gene in the same samples. All experiments were performed at least in triplicate in TM cultures from three donors.

**Protein Extraction and Western Blot Analysis**

Cells grown on 35-mm tissue culture dishes were washed twice with ice-cold PBS, collected, and lysed in RIPA cell lysis buffer. After centrifugation (19,000g for 30 minutes at 4°C) in a microfuge, the supernatants were transferred to fresh tubes and stored at −70°C for future use. The protein content was measured by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). Denatured proteins (2 μg) were separated under reducing conditions for p16 and p21 by electrophoresis on 7.5% SDS-polyacrylamide gels and for pRb by electrophoresis on 12% SDS-polyacrylamide gels. Thereafter, the proteins were transferred with semidry blotting onto a polyvinyl difluoride membrane (Roche Diagnostics) and probed with a rabbit polyclonal antibody against pRb (Ser 795, sc-21875; Santa Cruz Biotechnology), as described elsewhere.22 These antibodies were used at a dilution of 1:200. For detection, horseradish peroxidase-conjugated secondary antibodies were used at a 1:5000 dilution. Chemiluminescence was detected with an imager (LAS-1000; RayTest, Pforzheim, Germany). Exposure times ranged between 1 and 20 minutes. Quantification was performed on a computer (AIDA software; RayTest). All experiments were performed at least in triplicate in TM cultures from three donors.

**Statistical Analysis**

The results are expressed as the mean ± SD. For comparison of means between two groups, an unpaired t-test was used. Statistical significance was defined as P < 0.05.

**RESULTS**

**SA-β-Gal Activity**

Human TM cells were treated with 1.0 ng/mL TGF-β2 for 12, 24, and 48 hours. Untreated control cells kept for 12 hours showed 3.5% ± 0.6% senescence-associated SA-β-Gal-positive TM cells. Treatment with TGF-β2 for 12 hours had no effect on the number of SA-β-Gal-positive TM cells (2.7% ± 1.4%). Exposure to TGF-β2 for 24 and 48 hours markedly increased the proportion of SA-β-Gal-positive TM cells to 31.6% ± 7.7% and 33.7% ± 5.4% of total cells. Precinuciation of TM cells with the antioxidants ascorbic acid and coenzyme Q10 before 24 hours of TGF-β2 treatment reduced the number of SA-β-Gal-positive cells to 17.3% ± 7.4% and 22.5% ± 6.2% of total cells (Fig. 1). No major differences in the number of SA-β-Gal-positive cells were observed between passages 3 and 5 of different cell cultures (data not shown).

**TABLE 1. Primers Used for Real-Time PCR**

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<tr>
<th>Gene Target</th>
<th>Sequence Gene</th>
<th>Gene Position</th>
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<tr>
<td></td>
<td>5’-ggctctgctgtccttt-3’</td>
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<td>SM22</td>
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<td>185–202</td>
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<tr>
<td></td>
<td>5’-ctgtgtaaccagcttgctc-3’</td>
<td>259–278</td>
</tr>
<tr>
<td>SPARC</td>
<td>5’-ttggtctgtcgcctttg-3’</td>
<td>268–287</td>
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<td></td>
<td>5’-tggtgctctgccaagtttat-3’</td>
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**Protein Extraction and Western Blot Analysis of p16, p21, and pRb**

Cells grown on 35-mm tissue culture dishes were washed twice with ice-cold PBS, collected, and lysed in RIPA cell lysis buffer. After centrifugation (19,000g for 30 minutes at 4°C) in a microfuge, the supernatants were transferred to fresh tubes and stored at −70°C for future use. The protein content was measured by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). Denatured proteins (2 μg) were separated under reducing conditions for p16 and p21 by electrophoresis on 12% SDS-polyacrylamide gels and for pRb by electrophoresis on 7.5% SDS-polyacrylamide gels. Thereafter, the proteins were transferred with semi-dry blotting onto a polyvinyl difluoride membrane (Roche Diagnostics) and probed with a rabbit polyclonal antibody against p16 (C-20, sc-668; Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal antibody directed against p21 (DCS60; Cell Signaling Technology, Boston, MA), and goat polyclonal antibody against pRb (Ser 795, sc-21875; Santa Cruz Biotechnology), as described elsewhere.22 These antibodies were used at a dilution of 1:200. Chemiluminescence was detected with an imager (LAS-1000; RayTest, Pforzheim, Germany). Exposure times ranged between 1 and 20 minutes. Quantification was performed on a computer (AIDA software; RayTest). All experiments were performed at least in triplicate in TM cultures from three donors.
Lipid Peroxidation
In our experiments, lipid peroxidation of the cytoplasmic membrane of cultured TM cells was assessed by increased loss of PNA fluorescence. The PNA fluorescence of untreated cells kept for 48 hours was set to 100%. We observed a decrease in PNA fluorescence after TGF-β2 treatment for 12, 24, and 48 hours. The most significant decrease (to 80.3% ± 8.6%) was observed after 24 hours of TGF-β2 treatment. Preincubation of TM cells with the antioxidants ascorbic acid (AA) and coenzyme Q10 (Q10) on the number of SA-β-Gal-positive cells after 24 hours of TGF-β2 treatment were determined. Data (mean ± SD) are based on the sampling of 6 to 10 photomicrographs per condition from nine experiments with three different cell cultures from different donors (*P < 0.05). Co, control.

mRNA Expression of Senescence-Associated Biomarkers
Human TM cells were treated with 1.0 ng/mL TGF-β2 for 12, 24, and 48 hours. The signals generated by real-time PCR analysis in untreated control cells were set to 100%. Treatment with TGF-β2 for 12, 24, and 48 hours increased the Apo J mRNA expression by 1.5 ± 0.1, 2.2 ± 0.2, and 2.3 ± 0.1-fold (Fig. 3A) compared with that in untreated control cells (Fig. 2). No major differences in PNA fluorescence were observed in different cell cultures or between the third and fifth passages (data not shown).

FIGURE 1. TGF-β2 induced SA-β-Gal activity in cultured human TM cells. (A) Morphology and SA-β-Gal activity in untreated human TM cells. Only single cells stained blue, indicating SA-β-Gal activity. (B) In contrast, TM cells of the same passage exposed to TGF-β2 showed a marked increase of SA-β-Gal activity after 48 hours. (C) Quantification of the number of SA-β-Gal-positive cells. The percentage of SA-β-Gal activity was analyzed after 12, 24, and 48 hours of TGF-β2 treatment scored by counting at least 300 cells in phase-contrast photomicrographs of representative fields. In addition, the effects of the antioxidants ascorbic acid (AA) and coenzyme Q10 (Q10) on the number of SA-β-Gal-positive cells after 24 hours of TGF-β2 treatment were determined. Data (mean ± SD) are based on the sampling of 6 to 10 photomicrographs per condition from nine experiments with three different cell cultures from different donors (*P < 0.05). Co, control.

FIGURE 2. TGF-β2 increased lipid peroxidation. PNA fluorescence was analyzed after 12, 24, and 48 hours of TGF-β2 treatment. In addition, the effects of the antioxidants ascorbic acid (AA) and coenzyme Q10 (Q10) on PNA fluorescence after 24 hours of TGF-β2 treatment were examined. Data are expressed as the ratio of change compared with untreated control cells kept for 48 hours and represent the mean ± SD of results of nine experiments with three different cell cultures from different donors (*P < 0.05). Co, control.

Discussion
There is a growing body of evidence suggesting that an accelerated ageing process takes place in the aqueous humor outflow pathways in POAG.5,6,27–29 Previous morphologic investigations have also demonstrated a fourfold increased proportion of SA-β-Gal-positive TM cells of POAG donor eyes compared with age-matched control eyes.4 It is well recognized that oxidative stress plays the key role in the induction of SIPS.6,12 However, another factor that is also involved in the promotion of SIPS is TGF-β.14,15 In human diploid fibroblasts, untreated control cells (Fig. 3C). The TGF-β2-mediated increase of all three biomarkers was attenuated by antioxidants. Preincubation with ascorbic acid before TGF-β2 exposure led to a decreased induction of mRNA levels of Apo J, SM22, and SPARC, to approximately 1.4 ± 0.2, 1.2 ± 0.5, and 1.2 ± 0.3-fold compared with untreated control cells. A marked reduction of mRNA expression levels of Apo J (1.6 ± 0.3-fold), SM22 (1.3 ± 0.3-fold), and SPARC (1.1 ± 0.1-fold) after 24 hours of TGF-β2 treatment was also seen after preincubation with coenzyme 10 (Fig. 3).
for example, TGF-β induces SA-β-Gal activity and increases the mRNA levels of senescence-associated genes. Increased levels of TGF-β2 have also been assumed to be responsible for the pathologic changes of the TM of POAG eyes. In this study, we showed for the first time that treatment of cultured human TM cells with TGF-β2 led to a significant increase of the proportion of SA-β-Gal–positive cells. A similar effect was also observed in human TM cells after exposure to hydrogen peroxide (H2O2). One common characteristic of both TGF-β and H2O2 is the production of ROS. In our experiments in cultured human TM cells, we showed an increased lipid peroxidation that we estimated by determining the loss of PNA fluorescence after TGF-β2 treatment. Therefore, we can conclude that TGF-β2 is also capable of producing ROS in human TM cells.

To investigate whether ROS production is involved in TGF-β2–mediated senescent changes, we preincubated TM cells with antioxidants before TGF-β2 treatment. Preincubation with ascorbic acid and coenzyme Q10 reduced the TGF-β2–induced lipid peroxidation and thus the ROS release by TGF-β2. In addition, Apo J, SM22, and SPARC served as senescence-associated biomarkers. Apo J is a cellular chaperone that is inducible by TGF-β during SIPS. The senescence-associated proteins SM22 and SPARC are assumed to be involved in ECM turnover. Previous investigations have demonstrated that SM22 and SPARC can also be triggered by TGF-β during SIPS. In our study, TGF-β2 increased the expression of all three senescence-associated biomarkers, which was attenuated by preincubation with antioxidants. Furthermore, the number of SA-β-Gal–positive cells after TGF-β2 treatment was also decreased by antioxidants. However, antioxidants did not reduce the number of SA-β-Gal–positive cells to the levels of untreated control cells. These results suggest that TGF-β2–mediated ROS production may be involved at least in part in SIPS in cultured human TM cells. Previously, the role of TGF-β2 in POAG has been mainly defined by its induction of ECM in the outflow pathways, thus leading to increased outflow resistance. Our results support the hypothesis that elevated levels of TGF-β2 in POAG can also contribute to senescent changes in the TM. Senescent cells are involved in pathologic events such as production of ROS or accumulation of ECM. Whether TGF-β2–induced senescent TM changes are responsible for the increased outflow resistance and thus ele-
vate intraocular pressure, as observed in POAG, awaits further investigation.

From the studies of various cellular systems, it is known that stress-induced cellular senescence is mediated via two major signal transduction pathways, the p16-pRb and p21-pRb pathways, leading to the hypophosphorylation of the pRb protein.11–13 TGF-β induces both p16 and p21 in various senescent cell types.42,43 In this study, we showed that TGF-β increased the expression of p16, but had no effect on p21. Parallel to the upregulation of p16, we observed a downregulation of the pRb protein. Therefore, the effects of TGF-β may be associated with an increase in p16 and a decrease in pRb, which is the likely mechanism of action of TGF-β in the senescence response of human TM cells. Previous studies on human TM cells of old-aged donor eyes have detected an increased expression of both p16 and p21 compared with TM cells of younger-aged donor eyes.27 However, although the p21 senescence pathway is mainly activated during replicative senescence,44,45 the p16 senescence pathway constitutes the major mechanism in SIPS15,46,47 and may explain the activation of the p16-pRb pathway in TGF-β2-treated TM cells. In glaucomatous eyes, TM cells are characterized by low rates of cell division and chronic stress exposure.48 Whether p16-pRb pathway is the major signal transduction pathway in senescence-associated TM changes in POAG patients is yet unknown.

In summary, our in vitro experiments showed that TGF-β2 induced senescence-associated changes in human TM cells (Fig. 6). These senescence-related TM changes may lead to the loss of cell and tissue functionality in the outflow pathways of POAG patients. Therefore, it may be speculated that targeting cellular events leading to cellular senescence of the TM help to lower age-related changes in the TM as observed in glaucomatous eyes.

**References**
