Original Article
Effects of transforming growth factor-β2 on myocilin expression and secretion in human primary cultured trabecular meshwork cells

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Abstract: High intraocular pressure (IOP) is a risk factor for primary open-angle glaucoma (POAG). The trabecular meshwork (TM), a reticular tissue in the outflow passage of the aqueous humor (AH), is a major contributor to intraocular outflow resistance. High levels of myocilin (MYOC), which is expressed in the TM, are associated with high IOP. Furthermore, transforming growth factor-β2 (TGF-β2) concentrations in human AH are significantly elevated in POAG patients. This study was designed to investigate the effects of TGF-β2 on MYOC expression and secretion in human primary cultured TM cells. Primary cultured human TM cells were treated with 0 (control group), 1, 10, and 100 ng/mL TGF-β2 for 12, 24, or 48 h. MYOC mRNA and protein expressions in TM cells and protein secretion in conditioned media were analyzed by semi-quantitative RT-PCR, Western blotting, and enzyme-linked immunosorbent assays (ELISA), respectively. TM cells treated with 1, 10, and, 100 ng/mL TGF-β2 for 48 h showed higher MYOC mRNA and protein expressions than those in the control group (0 ng/mL TGF-β2) (all \( P < 0.05 \)). Treatment with TGF-β2 for 48 h also induced MYOC secretion in conditioned media in a dose-dependent manner (0 ng/mL: 7.107±1.163 pg/ml; 1 ng/mL: 7.879±1.894 pg/ml; 10 ng/mL: 8.063±1.181 pg/ml; 100 ng/mL: 8.902±0.699 pg/ml; all \( P < 0.05 \)). In Conclusion, TGF-β2 induced MYOC expression and secretion in human primary cultured TM cells. Further investigations are required to confirm the involvement of these two factors in POAG pathogenesis.

Keywords: Primary cell culture, trabecular meshwork cells, glaucoma, transforming growth factor-beta 2, myocilin

Introduction

Glaucoma is the second most prevalent cause of blindness in the world [1-5]. It is characterized by abnormally elevated intraocular pressure (IOP), chronic, progressive optic nerve injury, and visual field defects. Recent epidemiological surveys indicate that the incidence of primary open-angle glaucoma (POAG), a common type of glaucoma, is increasing in China [6, 7]. Although the pathogenesis of POAG remains unclear, it is commonly held that genetic and local environmental factors play important roles [8, 9]. High IOP is an important risk factor for POAG [10-12]; therefore, controlling IOP is the principal therapeutic approach. The trabecular meshwork (TM), a reticular tissue in the outflow passage of the aqueous humor (AH), is a major contributor to outflow resistance and is therefore a key focus of glaucoma research. Changes in TM structure and function may affect IOP [13, 14]. In patients with POAG, changes such as collagen and elastic fiber degeneration, increased extracellular matrix (ECM) deposition, cell shedding, and mesh narrowing or occlusion have been reported [15]. Such structural changes ultimately lead to increased AH outflow resistance and increased IOP.

Alterations in the concentration of a variety of cytokines have been reported in the AH of patients with POAG, and these changes in expression partially account for structural changes in the TM [16]. Notably, high concentrations of transforming growth factor-β2 (TGF-β2) in the AH of POAG patients have been reported [17]. Altered expression of TGF-β2 is thought to underlie many of the changes in the TM that contribute to the development and progression of glaucoma [18, 19]. These changes occur both intracellularly, within the actin cyto-
skeleton, and extracellularly, within the ECM of the TM. The result of elevated TGF-β2 is an increase in the outflow resistance of the TM brought about by modulation of the contractile properties of TM cells and of the composition and quantity of the TM ECM.

The myocilin gene (MYOC), which is also known as the TM glucocorticoid-induced response protein (TIGR) gene [20], is the first gene to be linked to POAG. To date, almost 100 disease-causing mutations have been characterized in the MYOC gene (see www.myocilin.com) [21]. The protein encoded by MYOC, which is highly expressed in the TM [22], contains domains that exhibit significant homology to the myosin and olfactomedin proteins [23, 24]. Various studies have demonstrated that MYOC protein localizes to intracellular compartments and to the extracellular matrix and that it exists in glycosylated and non-glycosylated forms [24, 25]. Given this complex expression pattern, its biological functions in normal and glaucomatous eyes have been difficult to elucidate. Findings published by a number of labs indicate that MYOC binds Ca2+ in the olfactomedin domain, displays molecular chaperone activity, participates in receptor-mediated endocytosis of a G-protein coupled receptor, and stimulates cell migration in a manner similar to that of Wnt3a [26-29]. MYOC interacts with itself and with a diverse array of proteins. These include extracellular matrix components such as collagens, laminin, fibronectin, and members of the secreted protein acidic and rich in cysteine (SPARC) family as well as with glyceraldehyde 3-phosphate dehydrogenase, alpha1-synthropin, gamma-synuclein, and myosin regulatory light chain [30-34]. The majority of disease-causing mutations in MYOC lie within the olfactomedin domain and are known to display altered intracellular sequestration, altered levels of secretion, or increased aggregation [35, 36]. Collectively, these and other data indicate that POAG caused by mutations in MYOC may result primarily from protein misfolding or improper protein trafficking.

Patients with mutations in MYOC account for only a small percentage of individuals with POAG. Howell and colleagues detected elevated levels of MYOC in approximately 70% of AH samples from 29 patients with POAG [37]. This finding indicates that MYOC expression may be associated with disease pathology in patients with POAG who lack MYOC mutations. Moreover, recent research suggests that MYOC protein can influence the organization and function of the TM and lead to elevated IOP [38]. Therefore, wild-type MYOC, like TGF-β2, may participate in the pathogenesis of some forms of POAG [39, 40]. However, the mechanisms underlying the effects of these two proteins remain to be elucidated, as does their regulatory interactions. In this study, TM cells from the eyes of non-glaucomatous donors were cultured in vitro to investigate the effects of TGF-β2 on MYOC expression and secretion. The results from the present study could provide new knowledge on the pathogenesis of POAG.

Materials and methods

Cell culture

TM tissues were obtained from four human eye donors (all of whom died in accidents; 2 women, aged 38 and 43 years, whose eyes were processed within 10 and 12 h, respectively, after their deaths; and 2 men, aged 35 and 40 years, whose eyes were processed within 12 and 15 h, respectively, after their deaths) who did not have any diagnosed eye disorder. Ethical approval was obtained from the Ethics Committee of Fujian Medical University, Quanzhou, China, and informed consent was obtained from the family members of the donors. The eyes were carefully dissected and divided into 3-5-mm fragments. The fragments were explanted into 25-cm2 disposable plastic tissue culture flasks and cultured in Dulbecco’s modified Eagle’s medium (Hyclone, Logan, UT, USA) containing 20% fetal bovine serum (Invitrogen-Gibco, Carlsbad, CA, USA) in an incubator set at 37°C and 5% CO2. This procedure resulted in the establishment of four cell lines, with one line being generated from one eye of one donor. All of the experiments were conducted in accordance with the tenets of the Declaration of Helsinki.
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**Cell identification**

Trabecular meshwork cells (third passage) were identified by transmission electron microscopy and immunocytochemical analysis. After centrifugation (800 rpm, 5 min), the cells were fixed with a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde in preparation for transmission electron microscopy (Hitachi H-7650, Tokyo, Japan) observation or grown on 25x25 mm cover slips in 6-well plates. At confluence, the cells were fixed in 4% paraformaldehyde for 1 h and goat anti-mouse IgG (all from Boster Company, Wuhan, China). Immunocytochemical staining (with a kit purchased from Boster) of LN, FN, NSE, Factor VIII-associated antigen, and vimentin were visualized with a Nikon Eclipse TE2000-U inverted fluorescent microscope (Tokyo, Japan).

**TM cell treatment with TGF-β2**

Trabecular meshwork cells (third passage) were grown to a density of 5×10⁶ cells/mm² in 6-well plates and incubated with serum-free DMEM for 24 h before drug treatments. The cells were then randomly divided into four groups and treated with recombinant human TGF-β2 (Peprotech, USA), which was dissolved in sterile distilled water to concentrations of 1, 10, and 100 ng/mL. Distilled water was used as the 0 ng/mL control solution. After incubation for 12, 24, or 48 h, culture media were collected and centrifuged for 20 min (2,000 rpm), and the supernatants were carefully extracted for analysis by enzyme-linked immunosorbent assay (ELISA). Adherent cells were prepared for RT-PCR and Western blot analysis of MYOC mRNA and protein expression, respectively.

**ELISA**

To determine whether TGF-β2 influences the secretion of MYOC by TM cells, ELISAs were performed according to the kit instructions (R&D Systems, Minneapolis, MN, USA). The standard solution was diluted (concentration gradient: 60 pg/ml, 40 pg/ml, 20 pg/ml, 10 pg/ml, 5 pg/ml, respectively) and 50 μL was added to each well. At the same time, 40 μL sample diluent was added to 10 μL culture supernatant from the TM cells treated with or without TGF-β2 or an equal volume of culture medium for blank samples. These samples were added and the plate was incubated at 37°C for 30 min before being washed and then dried. Then, 50 μL ELISA reagents were added to all wells and the plate was incubated at 37°C, washed, and dried again. Chromogenic reagents A and B (50 μL of each) were mixed gently and added to each well. The plate was incubated in the dark at 37°C for 15 min before 50 μL stop solution was added to each well to terminate the color reaction. The absorbance of each well was measured at 450 nm using a microplate reader (KHB ST-360, Shanghai, China).

**RT-PCR**

Total RNA was isolated with TRIzol according to manufacturer’s instructions (Beyotime Corporation, Jiangsu, China). RNA purity was determined by measuring the absorbance at 260 and 280 nm (A260/280), and the integrity of the RNA was verified by electrophoresis on formaldehyde gels. Reverse transcription was performed to obtain first-strand cDNAs by using an RT kit (Beyotime) according to the protocol. β-actin was used as an internal reference. Primer pairs for amplification of MYOC and β-actin were designed and synthesized by Sangon Co. Ltd. (Shanghai, China) as follows: MYOC: sense 5'-CCATTCAAGAACCGCTAT-3' and antisense 5'-GAAATTGTCTACGCCCTC-3'; β-actin sense 5'-CGGCTACAGCTTCACCAC-3' and antisense 5'-GTACTTGCGCTCAGGAGG-3'. PCR amplification was performed with pre-denaturation for 3 min at 94°C, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min, with a final extension for 10 min at 72°C. The PCR products were visualized by 1.5% agarose gel electrophoresis and photographed with a gel electrophoresis image analyzer (Bio-Rad, Hercules, CA, USA). The relative gray values (ODMYOC/ODβ-actin) represented relative expression levels of MYOC mRNA.

**Western blot analysis**

TM cells were rinsed with cold phosphate-buffered saline (PBS) solution, and RIPA lysis buffer (Beyotime) containing 100 mM phenylmethyl...
sulfonylfluoride (Beyotime) was added to extract the total proteins. All steps were carried out on ice when possible. Then, the lysates were centrifuged (12,000 rpm) for 20 min at 4°C. The total protein in the supernatant of each group was determined with a bicinchoninic acid assay (BCA) kit (Beyotime). A total of 30 μg protein from each group was used for 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the proteins were transferred to 0.2-mm pore nitrocellulose (NC) membranes (Millipore, MA, USA) that were then immersed in 5% skimmed milk dissolved in Tris-buffered saline containing 0.02% Tween-20 (TBST) overnight at 4°C. Then, the membranes were washed in TBST, incubated overnight at 4°C in blocking buffer containing 200 μg/mL mouse polyclonal anti-myocilin (R&D Systems, Minneapolis, MN, USA) and mouse monoclonal anti-β-actin (Biomiga, USA) antibodies. After washing in TBST, the membranes were incubated for 3 h at room temperature with 0.8 mg/mL horseradish peroxide-conjugated goat anti-mouse IgG (Zhongshan, Beijing, China) diluted in blocking buffer, and then washed in TBST. The blots were visualized with an ECL kit (Zhongshan, Beijing, China). After exposure for 2 min, the images were collected and analyzed by using a gel electrophoresis image analysis system. Relative MYOC protein expression was normalized to that of β-actin.

Statistical analysis

All statistical evaluations were conducted using a statistical software package (SPSS11.5, Chicago, IL, USA). Data are presented as the means±standard error of the mean (SEM) from the four cell lines with one cell line conducted in triplicate and were evaluated for normality and variance homogeneity. One-way analysis of variance (ANOVA) was employed to test differences among multiple groups, followed by the Student-Newman-Keuls (SNK) post-hoc test. If the tests indicated deviations from the para-
metric assumptions, the data were analyzed with the Kruskal-Wallis H test. $P < 0.05$ was considered to be statistically significant.

Results

TM cell identification

Donor eyes were processed as described in Materials and Methods. Four cell lines were established, with one cell line being generated from one eye of one donor. The cultured cells had various shapes that were stelliform or irregular. Each had processes [3-5] and obvious cell protrusions (Figure 1A). The nuclei were oval with clear membranes, and two or three prominent nucleoli were also visible. The cells were also hypertrophic with abundant transparent cytoplasm and a large number of phagocytosed black particles. Transmission electron microscopy showed that the cells were oval or round, with cell surface microvilli. Gap and tight junctions were the major connections between cells, and abundant organelles including lysosomes, endoplasmic reticulum, mitochondria, phagocytic vesicles, and ribosomes were visible in the cytoplasm (Figure 1B). Immunocytochemistry revealed that the cells expressed LN, FN, vimentin, and NSE, but did not express factor VIII-associated antigen (Figure 1C-G). These morphological and immunocytochemical results confirm that the cultured cells were TM cells [2-5].

Effect of TGF-β2 on MYOC mRNA expression in TM cells

We used RT-PCR to analyze the expression of MYOC mRNA in TM cells after treatment with different concentrations of TGF-β2. The expression levels, described as the ratio of the gray values of MYOC and β-actin, were $0.532\pm0.058$, $0.625\pm0.035$, $0.720\pm0.018$, and $0.828\pm0.024$ after treatment with 0, 1, 10, and 100 ng/ml TGF-β2, respectively, for 48 h (Figure 2). The relative values of MYOC mRNA in all of the experimental groups were significantly higher than that in the control group (0 ng/mL TGF-β2; $P < 0.05$). The expression of MYOC mRNA following TGF-β2 treatment increased in a dose-dependent manner. MYOC expression in cells exposed to TGF-β2 for 12 or 24 h was not significantly different from that of control cells (data not shown).

Effect of TGF-β2 on MYOC protein expression in TM cells

The ratios of the gray values of MYOC and β-actin in each group were $0.759\pm0.039$, $0.809\pm0.015$, $0.939\pm0.028$, and $1.069\pm0.011$ after treatment with 0, 1, 10, and 100 ng/ml TGF-β2, respectively, for 48 h (Figure 3). The relative values of MYOC protein in TM cells treated with 10 and 100 ng/ml TGF-β were both significantly higher than that in the control group ($P < 0.05$). The expression of MYOC protein following TGF-β2 treatment increased in a dose-dependent manner. MYOC expression in cells exposed to TGF-β2 for 12 or 24 h was not significantly different from that of control cells (data not shown).

Effect of TGF-β2 on MYOC protein secretion in TM cells

The results of analysis with ELISA showed that the concentrations of MYOC protein in the cul-
The concentration of TGF-β2 in human AH is significantly elevated in patients with POAG [17, 43]. The results of our study provide further evidence in support of the speculation that TGF-β2 may be involved in the pathogenesis of POAG. In the human TM, TGF-β2 enhances the expression of the matrix metalloproteinase-2 (MMP-2) precursor but reduces the activity of MMP-2 by enhancing the activity of plasminogen activator inhibitor-1 (PAI-1) [44]. As a result, exposure to TGF-β2 decreases degradation of the TM ECM, and this effect contributes to greater AH outflow resistance and increased IOP. These and other data indicate that TGF-β2 can trigger a complex process of remodeling within the TM ECM. How might MYOC contribute to this event? MYOC interacts with itself and with a number of ECM proteins, such as collagen, fibronectin, and laminin [33]. Additionally, analysis in mice that overexpress MYOC revealed altered expression of two proteins involved in cell adhesion and signaling: the carcinoembryonic-antigen-related celladhesion molecule (CEACAM) and mindin/spondin 2 [45]. Taken together, the available evi-

**Figure 3.** Effect of TGF-β2 on the expression of MYOC protein in human primary cultured TM cells. TM cells were treated with 0, 1, 10, and 100 ng/ml TGF-β2, respectively, for 48 h. Relative MYOC protein expression was determined by Western blot. β-actin was used as an internal reference. The data are shown as the means ±SEM from the four cell lines with one cell line conducted in triplicate. *P < 0.05 vs. 0 ng/ml; #P < 0.05 vs. 1 ng/ml; ΔP < 0.05 vs. 10 ng/ml.
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Figure 4. Effect of TGF-β2 on the secretion of MYOC protein in human primary cultured TM cells. TM cells were treated with 0, 1, 10, and 100 ng/ml TGF-β2, respectively, for 48h. The secretion of MYOC protein was determined by ELISA. The data are shown as the mean±SEM from the four cell lines with one cell line conducted in triplicate. *P < 0.05 vs. 0 ng/ml; #P < 0.05 vs. 1 ng/ml; ΔP < 0.05 vs. 10 ng/ml.

Our observations are in conflict with the data reported by Howell et al. [37], who found no significant correlation between myocilin secretion and TGF-β2 levels in patients with POAG. However, this study was performed in a small number of patients and deserves further investigation. Our findings also differ from those of Resch et al [51]. These authors treated cultures of primary human TM cells with 10, 100, or 1000 ng/ml of TGF-β2 but did not observe increased secretion of MYOC in conditioned media. We believe that this discrepancy arises from differences in experimental procedures. First, Resch and colleagues used Western blots to analyze the expression of MYOC in the conditioned media of TM cells treated with TGF-β2, while we used ELISA. Western blotting and ELISA analyses differ in sensitivity and in the nature of quantitative data that is obtained. Although not large in magnitude, we observed significant differences in the quantity of MYOC protein in the media of TM cells after exposure to TGF-β2. Importantly, we complemented our examination of the expression of secreted MYOC protein with analyses of the expression of intracellular MYOC protein and MYOC mRNA, and our results support the finding that treatment with TGF-β2 increases the secretion of MYOC in the TM. Second, Resch et al. report that the TM cells used in their experiments were passaged four to eight times, while the cells used in our experiments were at the third passage. The possibility exists that extended passage of TM cells leads to a different response profile after exposure to TGF-β2. After consideration of these factors, we conclude that regulation of MYOC by TGF-β2 in vitro is sensitive to experimental conditions and that additional studies of the relationship between these two proteins must account for this sensitivity.

Based on the results of this study, we conclude that TGF-β2 promotes the expression and secretion of MYOC in human primary cultured TM cells. However, further research is needed to determine the signaling events that include
these two factors in the pathogenesis of POAG. An improved understanding of the etiology of POAG is essential for diagnosis and the development of therapeutic strategies.

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Disclosure of conflict of interest

None.

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References

[19] Prendes MA, Harris A, Wirostko BM, Gerber AL and Siesky B. The role of transforming growth factor beta in glaucoma and the therapeutic
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