·Basic Research·

Interference of Y-27632 on the signal transduction of transforming growth factor beta type 1 in ocular Tenon capsule fibroblasts

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Abstract

• AIM: To investigate the interfering effect of Y-27632, a ROCK-I selective inhibitor, on the signal transduction pathway of transforming growth factor-β 1 (TGF-β 1) in ocular Tenon capsule fibroblasts (OTFS) *in vitro*.

• METHODS: After OTFS from passages 4 to 6 *in vitro* were induced by TGF-β 1 and then treated by Y-27632, the changes of the OTFS cell cycles were analyzed *via* flow cytometry, and the proteins expression of the α -smooth muscular actin (α -SMA), connective tissue growth factor (CTGF), collagen I were calculated by Western blot. After OTFS treated by the different concentrations of Y-27632, the expression levels of the α -SMA, CTGF and collagen I mRNA were assayed by RT-PCR.

• RESULTS: Y-27632 had no markedly effect on the OTFS cell cycles. After treated by TGF-β 1, OTFS in G1 period significantly increased. The cell cycles distribution by both TGF-β 1 and Y-27632 had no remarkable difference from that in control group. Y-27632 significantly inhibited the proteins expressions of both α -SMA and CTGF, while to some extent inhibited that of collagen I. TGF-β 1 significantly promoted the proteins expressions of α -SMA, CTGF and collagen I. After OTFS treated by both TGF-β 1 and Y-27632, of α -SMA, the protein expression was similar with that in control group (P=0.066>0.05), but the protein expression of CTGF or collagen I, respectively, was significantly different from that in control group (P=0.000<0.01). The differences of expressions of the α -SMA, CTGF and collagen I mRNA in 30, 150, 750μ mol/L Y-27632 group were statistically significant, compared with those in control group, respectively (α -SMA, P=0.002, 0.000, 0.000; CTGF, P=0.014, 0.002, 0.001; collagen I, P=0.003, 0.002, 0.000).

• CONCLUSION: Blocking the Rho/ROCK signaling pathway by using of Y-27632 could inhibit the cellular proliferation and the expression of both CTGF and α -SMA whatever OTFS induced by TGF-β 1 or not. Y-27632 suppressed the expression of collagen I mRNA without induction.

• KEYWORDS: Y-27632; ocular Tenon's capsule fibroblasts; transforming growth factor beta type 1; α -smooth muscular actin; connective tissue growth factor; collagen I

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INTRODUCTION

The main cause of failure of glaucoma filtration surgery is postoperative scarring in the filtering bleb. Fibroblasts from the subconjunctival space play a key role in the scarring process. Perioperative administration of antimetabolites such as 5-fluorouracil and mitomycin C (MMC) is effective in limiting the scarring process, but accompanied by severe side effects [1].

Rho is known to function as a molecular switch in various cellular functions including formation of stress fibers and focal adhesions [2], regulation of G1 to S cell cycle progression. ROCK I, one of the putative target molecules of Rho, has been identified as a Rho effector. ROCKs are important for regulating focal adhesions and stress fiber formation in cultured fibroblasts and epithelial cells [3]. Transdifferentiation of fibroblasts into myofibroblasts is a crucial step in wound healing and scar formation, which is associated with expression of α-smooth muscle actin (α-SMA) [4]. Enhanced α-SMA expression indicates the

activation of Rho/ROCK signal channel in fibroblasts [5]. On the other hand, TGF-β1 in serum was known to be one of the most potent stimulating factors for fibroblast [6,7]. One important issue generally ignored was that, in the intraoperative and especially postoperative period of the trabeculectomy, OTFS had been constantly exposed to TGF-β1 originated from serum, duo to the damage of the blood-aqueous barrier and the filtering road immersed in the circulating aqueous[8,9].

In the present study, we hypothesized that in the process of scar formation, both Rho/ROCK and TGF-β1 signal channels would be mutually affected. Therefore, OTFS cultured *in vitro* and given exogenous TGF-β1, were to simulate the microenvironment of the OTFS fibrosis of the glaucomatous filtration road. By blocking the Rho/ROCK signaling pathway by administration of Y-27632, we tested Y-27632 ability to inhibit proliferation of OTFS induced by TGF-β1. We also investigated its effect on connective tissue growth factor (CTGF) and collagen I, the two downstream fibrosis factors of TGF-β1 signal channel. We eventually demonstrated that OTFS proliferation was ameliorated by Y-27632 administration along with the suppression of CTGF and extracellular collagen synthesis.

MATERIALS AND METHODS

Materials GENMED universal cell circle analysis kit of flow cytometry (Shanghai Jeramain gene meditechnology, China), BCA protein concentration assay kit (Jiangsu Biyuntian biotechnology, China), to rat anti-β-tubulin antibody (Sigma), to rat anti-α -SMA antibody (ab7817, Abcam), to rat anti-CTGF antibody (ab51704, Abcam), anti-collagen I antibody, (sc28657, SantaCruz), (HRP) -labeled secondary antibody (ab6789, ab6721, Abcam), RNAeasy kit (TaKaRa, Japan), gel imaging system (Image Master VDS, Pharmacia Biotech).

Methods

Culture of OTFS and cellular stimulation Small Tenon biopsy specimens were obtained during standard cataract surgery after selected patients had received comprehensive information and provided written consent for the procedure. The protocol was approved by the Institutional Review Board at Medical School of Xi'an Jiaotong University in compliance with tenets of the Declaration of Helsinki. Primary OTFS obtained from expansion cultures of the Tenon explants were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum and antibiotics. Cells were maintained in the logarithmic growth phase, and cells from passages 4 to 6 were used in all experiments, which were performed at least three times with similar results.

Analysis of cellular cycle alterations by flow cytometry OTFS cells (1×10^6) were plated onto a 6-well plate and rested overnight. Cells were incubated in DMEM containing 1% serum and randomly separated into 4 groups: blank control (without Y-27632 or TGFβ1), TGF-β1group (supplemented with 5ng/mL TGF-β1 for 24 hours), Y-27632 group (supplemented with 150μmol/L Y-27632 for 24 hours), and TGF-β1+Y-27632 group (supplemented with 5ng/mL TGFβ1 followed by additional treatment with 150μmol/L Y-27632 for 24 hours). Cells were resuspended by adding ice-cold PBS, centrifuged at 10 000r/mim twice, and mixed up with 600μL ethanol rapidly to fix cells (the final concentration of ethanol: 60%-70%). Cells were added with 1mL propidium iodide (the final concentration of 10μg/mL) and stained for 15 minutes photophobically. In accordance with the manufacturer's instructions of GENMED universal cell circle analysis kit of flow cytometry, cells cycle were measured up.

Western blotting OTFS cells, after treated samely as above and randomly divided into 4 groups, were lysed at 4℃ in RIPA buffer (50mmol/L Tris-HCL pH8.0, 150mmol/L NaCl, 1%NP-40, 0.1% SDS, 0.5% DOC, 2mmol/L EDTA, lmmol/L PMSF, 10μmol/L leupeptin, 1μmol/L pepstatinA, lμg/mL aprotinin) and extracts clarified at 10 000×g for 20 minutes. After separation on 10% SDS-PAGE, proteins were assessed using the BicinChoninic Acid assay BCA protein concentration assay kit. Aliquots (80μg cellular protein) were electrophoretically separated, transferred to nitrocellulose membranes, washed with Tris-buffered saline and Tween20, incubated overnight with specific antibodies (to rat anti-β-tubulin antibody, Sigma, monoclonal, 1:1000; to rat anti-α-SMA antibody, ab7817, Abcam, monoclonal, 1:100; to rat anti-CTGF antibody, ab51704, Abcam, monoclonal, 1:500, anti-collagen Ⅰ antibody, sc28657, SantaCruz, 1:150). After washing three times in 0.05% Tween20 in TBS, primary antibodies were detected with the horseradish peroxidase (HRP)-labeled secondary antibody (ab6789, ab6721, Abcam, 1:10000). Immunoreactive proteins were visualized with ECL reagent and quantitated by densitometry. Stripped membranes were quantified by Image-Pro Plus 6.0 (Image Software IPP6.0, Media Cybernetics, USA).

Real time (RT)-polymerase chain reaction (PCR) OTFS cells (1×10^6) were plated onto a 96-well plate, digested by 0.25% Trypsin, incubated in DMEM containing 1% serum and supplemented 6, 30, 150 and 750μmol/L Y-27632. Total RNA from OTFS cells was extracted (RNAeasy kit, TaKaRa, Japan) according to the manufacturer's instructions. RNA was quantitated spectrophotometrically at 260/280nm. Equal amounts of RNA were then reverse transcribed (Advantage RT-for-PCR kit, TaKaRa Clontech) according to the manufacturer's instructions in 10μL total system of the reverse transcription (2μL 5×primescript™ buffer, 0.5μL Oligo dT primers, 0.5μL primescript™ RT enzyme mix, 2μL RNA, 5μL deRNase). Real-time quantification of

α-SMA, collagen Ⅰ and CTGF expression in different concentrations of Y27632 treated OTFS cells was performed. cDNA from different treated samples were normalized with an endogenous housekeeping gene, β-tubulin. Primer sequences were for α-SMA: 5'-AAACAG GAATACGACGAAG-3' (forward) and 5'-CAGGAATGAT TTGGAAAGGA-3' (reverse), CTGF:5'-TTCCCCCAGCC ACAAAGAGTC-3' (forward) and 5'-GGGAGCCGAAGTC ACAGAAG-3' (reverse), Collagen I:5'-TTCCCCCAGCCA CAAAGAGTC-3' (forward) and 5'-CGTCATCGCACAAC ACCT-3', β-tubulin: 5'GAGCTGTTCAAGCGCATCTC-3' (forward) and 5'-TCCTCCTCGTCGTCTTCGTA-3' (reverse). The PCR master mix (SYBR Green PCR reagent kit, TAKARA) consisted of 1μL template cDNA, 1μL upstream primer, 1μL downstream primer, 10μL 2×Premix Ex Taq Ⅱ, 7μL ddH_2O in 20μL reaction. Duplicate PCR reactions were carried out using the following amplification protocol: 95℃ for 2 minutes followed by 40 cycles of 95℃ for 30 seconds, 95℃ for 5 seconds, and 60℃ for 20 seconds. PCR products were then subjected to 1.5% polyacrylamide gel electrophoresis with gel imaging system (Image Master VDS, Pharmacia Biotech) for detection. The housekeeping gene β-tubulin was amplified as an internal control for normalizing the cDNA content of control and test compound-treated samples in PCR reactions. Relative gene quantities were obtained using the comparative threshold (C_t) method.

Statistical Analysis All values were reported as the means and errors of the mean (SEM). Calculations were performed using the SPSS program software package (SPSS Software version 13.0). Multigroup comparisons were performed by one-way analysis of variance (ANOVA), followed by the Levene test as variance homogeneity analysis of each sample means (P>0.05). Differences for paired samples were analyzed by the LSD-t test.

RESULTS

Cellular Cycle Alterations Compared with 55.28% in G1 phase of OTFS cells of control group, 57.19% in G1 phase of OTFS cells of Y-27632 group signified that Y-27632, a specific inhibitor of Rho kinase, had no significant effect on OTFS cells cycle. TGF-β1 can positively and radically stimulate OTFS cells due to the fact that cells in G1 phase substantially increased up to 72.62% of TGF-β1 group. Co-treated by TGF-β1 and Y-27632, Y-27632 weakened the stimulating effect of TGF-β1 for OTFS cells with 53.03% in G1 phase (Figure 1).

Expressions of α-SMA, Collagen I and CTGF Protein 150μmol/L Y-27632 successfully blocked up the expression of α-SMA protein, the downstream factor of ROCK signal pathway and independently inhibited the expression of CTGF protein, the downstream factor of TGF-β1 signal pathway, which TGF-β1 significantly promoted. Co-treated

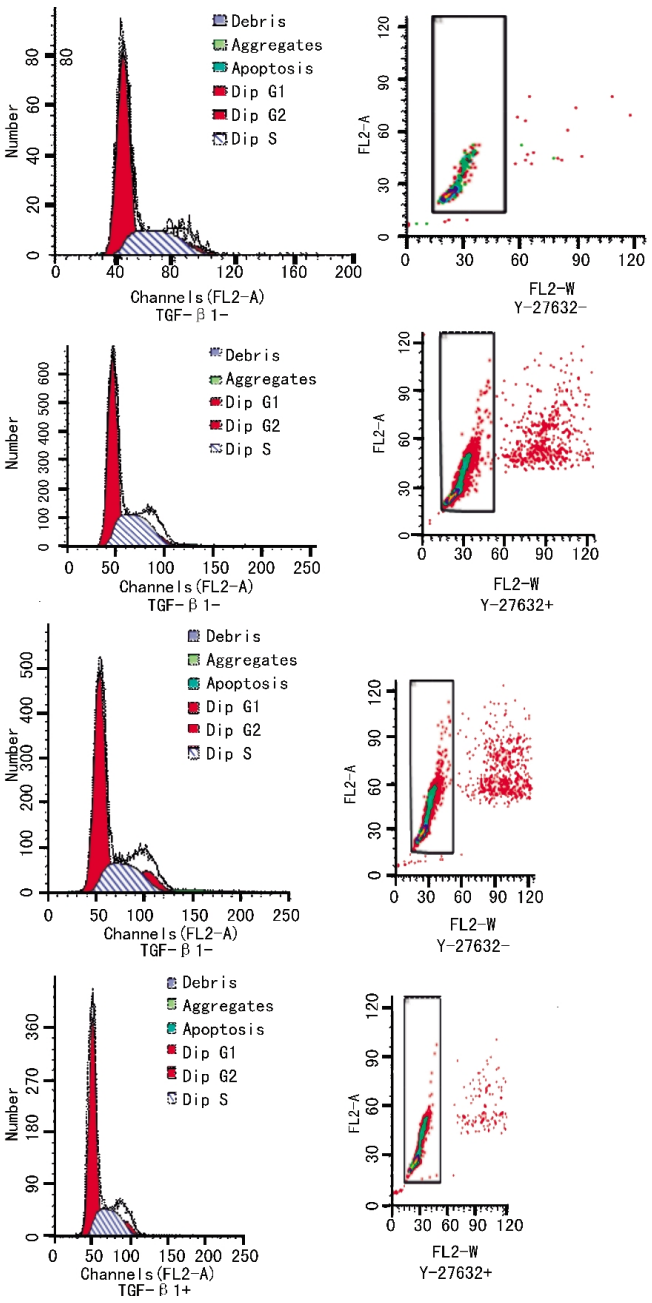

Figure 1 Analysis of cellular cycle alterations by flow cytometry.

by TGF-β1 and Y-27632, the ratio of the Integral Optical Density (IOD) for α-SMA (0.4252±0.1280) was statistically less than the IOD ratio in control group (0.4897±0.1243, Figure 2A, P=0.066>0.05). Similarly, the IOD ratio for CTGF (0.3341±0.0099) was significantly less than the IOD ratio in control group (1.1232 ±0.0072, Figure 2B, P= 0.000<0.01). Evidently, Y-27632 weakened up the expressions of both α-SMA and CTGF proteins of OTFS cells stimulated by TGF-β1. Moreover, Y-27632 inhibited CTGF expression more significantly than α-SMA protein.

The expression of collagen I protein was radically promoted by TGF-β1, and 150μmol/L Y-27632 had inhibitory effect. Y-27632 had no profound effect against or on the cellular synthesis of collagen induced by TGF-β1 since there was no statistical significance between the IOD ratio in co-treatment

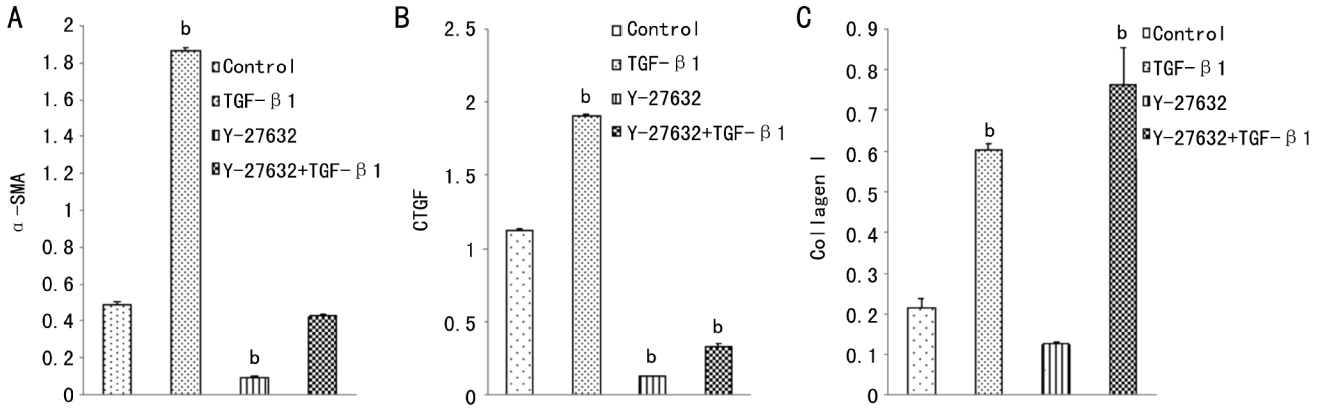

Figure 2 Expressions of α-SMA, collagen I and CTGF protein by Western blotting A: Effects of 150μmol/L Y-27632 or 5ng/mL TGF-β1 on the expression of OTFS-mediated α-SMA protein. $^bP<0.01$ *vs* control; B: Effects of 150μnol/L Y-27632 or 5ng/mL TGF-β1 on the expression of OTFS-mediated CTGF protein. $^bP<0.01$ *vs* control; C: Effects of 150μmol/L Y-27632 or 5ng/mL TGF-β1 on the expression of OTFS-mediated collagen I protein. $^bP<0.01$ *vs* control.

Table 1 The Gene Expressions of α-SMA, Collagen I and CTGF

IOD ratio	0μmol/L	6μmol/L	30μmol/L	150μmol/L	750μmol/L
α-SMA	1.9937±0.4513	1.7356±0.0494	1.2744±0.1077	0.9320±0.1490	0.6797±0.0315
CTGF	1.7628±0.3200	1.8451±0.0926	1.1858±0.0439	0.8748±0.0667	0.7650±0.0552
Collagen	2.3504±0.1613	1.9466±0.4994	1.3370±0.1274	1.2882±0.4637	0.6455±0.1447

group by TGF-β1 and Y-27632 (0.7598±0.9437) and that in TGF-β1 group (0.6044±0.1266, Figure 2C, $P=0.053>0.05$), (Figure 3).

Gene Expressions of α-SMA, Collagen I and CTGF In the experiment of the effect of different concentrations of Y-27632 on the expressions of α-SMA, collagen I and CTGF mRNA, there were statistical differences of 3 genes expressions respectively between different concentrations of Y-27632(30, 150, and 750μmol/L) and control group (Figure 4, $P<0.05$). 30, 150, and 750μmol/L Y-27632 not only significantly inhibited the expressions of α-SMA, collagen I and CTGF mRNA, but 3 genes expressions gradually declined as the dosage of Y-27632 rose up. Of OTFS cells stimulated by 6μmol/L Y-27632, the expressions of α-SMA, collagen I and CTGF mRNA altered insignificantly as compared to that in control group (Table 1).

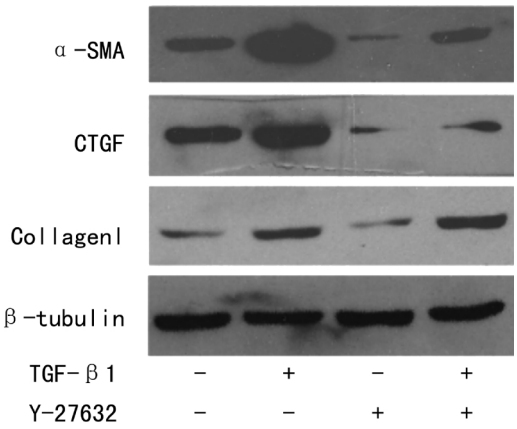

Figure 3 Gel electrophoresis bands TGF-β1 markedly made the bars of α-SMA, CTGF, and collagen I proteins thicker, especially for α-SMA, CTGF. Y-27632 made the bars of α-SMA and CTGF proteins thinner. After co-treated by TGF-β1 and Y-27632, the bars of α-SMA and CTGF proteins were similar to that in Y-27632 group. The band of collagen I protein generally altered insignificantly. β-tubulin was considered as internal reference.

DISCUSSION

TGF-β1 had the chemotactic effect and mainly in the early stage induced fibroblasts to migrate and proliferate [10,11]. In the cellular cycle experiment, Rho/ROCK signal blocking seemed insignificantly to affect the cell cycle of OTFS, but inhibited the role that TGF-β1 accelerated the cell cycle. When TGF-β1 made the cellular proportion in G1 phase markedly increase and the cellular proliferation accelerate, 150μmol/L Y-27632 offset the effect after OTFS had been interfered with TGF-β1 for 48 hours.

In the intermediate and late stage of wound healing, TGF-β1 chiefly induced the cellular phenotypes to transform, in which a variety of cell signal channels were involved [6,11]. ROCK signal channel regulated the cell migration through the phosphorylation of myosin light chain (MLC) to change the actin cytoskeleton so that α-smooth muscle actin was generally considered as one of the primary downstream substrates of ROCK signal channel[12-14].

According to our preliminary experiments, OTFs cytotoxicity happened when concentration of Y-27632 increased to 1000μmol/L. So concentrations of Y-27632 applied in this study were adjusted to 0, 6, 30, 150, 750μmol/L. In the experiment of α-SMA mRNA, 30, 150, 750μmol/L Y-27632 successfully suppressed Rho/ROCK signal channel and the gene expression of α-SMA decreased with the concentration

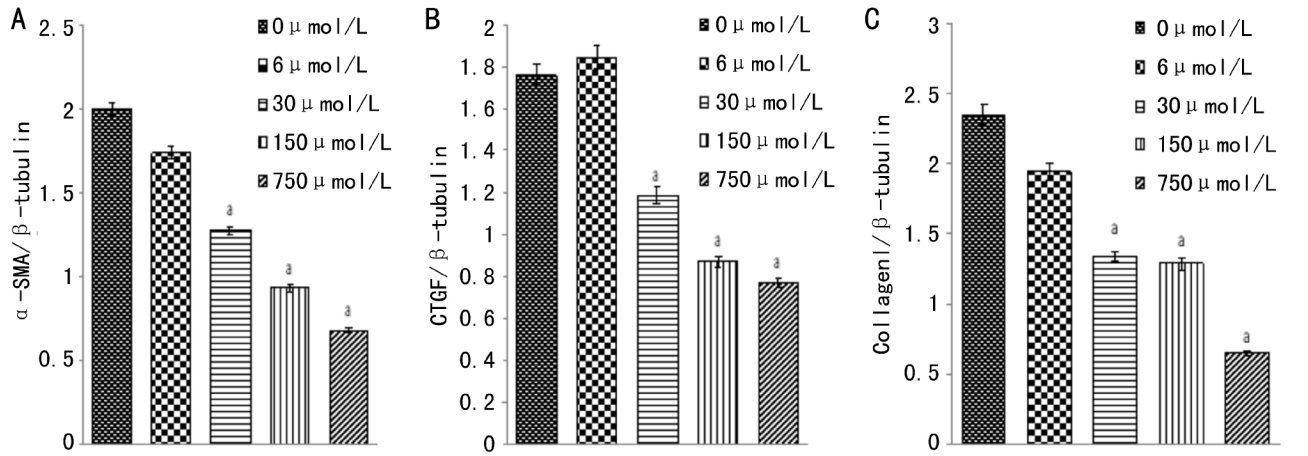

Figure 4 Expressions of α-SMA, collagen I and CTGF mRNA by real time PCR A: Effects of Y-27632 on the expression of OTFS-mediated α-SMA mRNA. $^{a}P<0.05$ *vs* control; B: Effects of Y-27632 on the expression of OTFS-mediated CTGF mRNA. $^{a}P<0.05$ *vs* control; C: Effects of Y-27632 on the expression of OTFS-mediated collagen I mRNA. $^{a}P<0.05$ *vs* control.

of Y-27632 increasing. 6μmol/L Y-27632 cannot inhibit the Rho/ROCK signal channel supposedly due to its low level of concentration. The gene expression of α-SMA of 6μmol/L Y-27632 was insignificantly different from that in control group. Hence, 150μmol/L Y-27632 was chosen to interfere in the TGF-β1 signal channel. In the experiment of α-SMA protein, 150μmol/L Y-27632 profoundly inhibited the expression of α-SMA protein. Conversely, TGF-β1 significantly promoted its expression, which signified TGF-β1 had the similar effect to Y-27632 to promote the actin cytoskeleton to change and speed up the OTFS migration. Y-27632 not only counteracted, but inhibited the effect that TGF-β1 enhanced the expression of α-SMA protein.

CTGF was known as the direct downstream effector of TGF-β1. CTGF and TGF-β1 promoted cell proliferation and extracellular matrix synthesis, mediated the cell adhesion and chemotaxis, induced the cell apoptosis, and promoted the angiogenesis. Demonstratively the expressions of CTGF mRNA and protein in hypertrophic scars and keloids increased, especially in keloids, compared with extremely low level of those in normal skins [15,16]. In the proliferative, slow down, and mature stage of hypertrophic scars the expression intensity of CTGF turned accordingly from strong to weak. It indicated the expression intensity of CTGF was consistent with tissues fibrosis degrees [16,17]. In the experiment of CTGF mRNA, 30, 150, 750μmol/L Y-27632 significantly suppressed the expression of CTGF mRNA and the gene expression of CTGF decreased with the concentration of Y-27632 increasing, although there was no statistical difference of the expression of CTGF mRNA between 150 and 750μmol/L Y-27632. Additionally 6μmol/L Y-27632 cannot change its expression. In the experiment of CTGF protein, after the Rho/ROCK signal channel blocked up, the synthesis of CTGF, the downstream effector of TGF-β1, was suppressed by Y-27632. 150μmol/L Y-27632 significantly inhibited the effect that TGF-β1 intensified the expression of CTGF protein though Y-27632 without TGF-β1 induction can inhibit the expression of CTGF protein more strongly.

TGF-β1 can promote the synthesis of the extracellular matrix, of which collagen was one of the major components [18,19] and collagen I constituted the main collagen of the scar. Thus collagen I was generally considered as one of the downstream effectors of TGF-β1. In the present study, similarly to CTGF, the expression of collagen I mRNA was obviously suppressed by 30, 150, 750μmol/L Y-27632, and the gene expression of collagen I decreased with the concentration of Y-27632 augmentation. Meanwhile no statistical difference of the expression of collagen I mRNA existed between 30 and 150μmol/L Y-27632, and 6μmol/L Y-27632 had no effect to change its expression. In the experiment of collagen protein, after the Rho/ROCK signal channel blocked up, the cellular collagen synthesis was suppressed. 150μmol/L Y-27632 statistically inhibited the expression of collagen I protein, while TGF-β1 significantly promoted its expression. However, blocking of the Rho/ROCK signal channel played an insignificant role in the TGF-β1 mediated collagen synthesis probably because the effect of TGF-β1 mediated collagen synthesis was so strong that Y-27632 cannot affect the signal channel of TGF-β1/collagen I.

Simple suppression of TGF-β1, which functions in wide range, can bring to serious consequences [20]. By inhibiting the expression of α-SMA, the downstream marker of the Rho/ROCK signal channel in OTFS, CTGF and collagen I, the downstream fibrosis effectors of TGF-β1 signal transduction, both had been suppressed at varying degree. Additionally, Y-27632 as a specific inhibitor of Rho kinase type 1, without TGF-β1 induction of OTFS, can effectively suppress both the proliferation of OTFS and the expression of fibrosis effectors. Hopefully, the Rho/ROCK signal

channel was selectively interfered to regulate or block the TGF-β1 signal transduction. This point was our research target for postoperative anti-scar treatment of glaucoma and helps us to have a deeper understanding for the mechanisms of the intracellular fibrosis in OTFS.

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