Extracellular matrix gene expression in human trabecular meshwork cells following mechanical fluid flow stimulation

Koichi Yoshida¹, Motofumi Kawai¹, Tsugiaki Utsunomiya¹, Akihiro Ishibazawa¹, Young-Seok Song¹, Mariana Sayuri B. Udo¹, Yoshikazu Tasaki², Akitoshi Yoshida¹

¹Department of Ophthalmology, Asahikawa Medical University, Asahikawa, Hokkaido 078-8510, Japan
²Department of Hospital Pharmacy & Pharmacology, Asahikawa Medical University, Asahikawa, Hokkaido 078-8510, Japan

Correspondence to: Koichi Yoshida. Department of Ophthalmology, Asahikawa Medical University, 2-1-1-1, Midorigaokahigasi, Asahikawa, Hokkaido 078-8510, Japan. yoshikou@asahikawa-med.ac.jp

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Abstract

● **AIM:** To investigate changes in extracellular matrix (ECM) gene expression in human trabecular meshwork (HTM) cells in response to mechanical fluid flow stimulation.

● **METHODS:** HTM cells were grown on a glass plate coated with 0.02% type I collagen (COL) and exposed to shear stress (0, 0.2, 1.0 dyne/cm²) for 12h. Changes in genes related to the ECM were evaluated by real-time reverse transcriptase-polymerase chain reaction. Phosphorylation of Smad2 protein was investigated by Western blotting.

● **RESULTS:** After mechanical stimulation, COL type 4 alpha 2, COL type 6 alpha 1, and fibronectin-1 mRNA were significantly higher than the static control (P<0.05, <0.05, and <0.01, respectively). The metalloproteinase-2 and plasminogen activator inhibitor-1 mRNA were significantly higher than the static control (P<0.05 and <0.01, respectively), while the differences in the tissue inhibitors of metalloproteinases-2 mRNA were not significant. The phosphorylation of Smad2 levels was significantly higher compared to the static control cells.

● **CONCLUSION:** Changes in the expressions of genes associated ECM metabolism result in HTM cells after mechanical stimulation. The mechanical stimulation of the aqueous humor to the trabecular meshwork may promote ECM turnover and contribute to intraocular pressure homeostasis.

● **KEYWORDS:** trabecular meshwork; shear stress; aqueous humor; extracellular matrix; glaucoma; intraocular pressure

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INTRODUCTION

The intraocular pressure (IOP) is maintained via a function of the aqueous humor, which is produced by the ciliary body¹. About 70% to 95% of the aqueous humor discharge via the conventional outflow pathway². Increased aqueous outflow resistance in this outflow pathway is the reason of glaucoma accompanied by elevated IOP³. Therefore, normal functioning of the outflow component is important to the IOP homeostasis and prevention of glaucoma⁴. The trabecular meshwork (TM), juxtacanalicular (JCT) meshwork, Schlemm’s canal (SC), collector channels, and episcleral veins comprise the conventional outflow pathway. Among those, the extracellular matrix (ECM) in the TM tissue, which is comprised of collagens (COL) or fibronectin (FN)⁵, is critical for the homeostatic maintenance of the normal outflow resistance⁶. Of note, recent studies have revealed that ECM turnover is regulated by mechanical stress, at least in part⁷. Experimentally, mechanical stretching of the TM cells up-regulates the matrix metalloproteinase-2 (MMP-2), the membrane type-1 MMP, and reciprocally down-regulates the tissue inhibitors of metalloproteinases (TIMP)-2⁸. Transcription of MMPs and proMMP activation is regulated by tissue plasminogen activator⁹, which knockout mice expressed in outflow facility reduction and declined the expression of MMP-9 gene in angle tissues¹⁰. Cyclic mechanical stress alters the contractility of the TM cells¹¹. Mechanistically, the mechanosensitive receptors on the TM cell surface reportedly sense the deformation of the ECM resulting from the IOP fluctuations via integrin-based cell-matrix contact¹². Collectively, these findings suggest that mechanical stress applied to the TM cells is important for the ECM turnover.
Clinically, it generally is considered that the TM function (e.g., ECM synthesis, phagocytosis, contractility) deteriorates after glaucoma filtration surgery. In addition, the aqueous outflow to the TM after filtration surgery decreases to about 10% of the preoperative level\(^\text{[13]}\). These facts cause speculation that the mechanical fluid flow stimulation by aqueous outflow to the TM also plays an important role in maintaining the TM function. However, to our knowledge, no previous reports have noted the difference of mechanical fluid flow stimulation on TM cells and its effect on ECM turnover, although the effects of mechanical stimulation by stretching of the TM cells have been investigated previously\(^\text{[14]}\).

In the current study, we applied mechanical fluid flow stimulation to the human trabecular meshwork (HTM) cells and investigated the gene changes related to the ECM and ECM remodeling.

**MATERIALS AND METHODS**

**Ethical Approval** The current study was conducted according to the tenets of the Declaration of Helsinki, and the study did not require ethical approval including those involving human-derived materials.

**Materials** Recombinant human transforming growth factor (TGF)-β2 was bought from R&D Systems (Minneapolis, MN, USA). Dexamethasone (DEX) and Y-27632 were bought from Merck Millipore (Darmstadt, Germany). Polyclonal myocilin (MYOC) antibody (rabbit, ab41552, 1:1000) was bought from Abcam (Cambridge, UK); monoclonal β-actin antibodies (mouse, #3700, 1:3000) were bought from Cell Signaling Technology (Danvers, MA, USA); polyclonal FN antibodies (rabbit, F3648, 1:1000) and polyclonal COL IV antibodies (rabbit, SAB4500369, 1:1000) were bought from Sigma-Aldrich (St. Louis, MO, USA).

**Cell Culture** The primary HTM cells obtained from ScienCell (cat. no. 6590, lot no. 3423, Carlsbad, CA, USA) were maintained in Fibroblast Medium (ScienCell) containing 5% fetal bovine serum and fibroblast growth supplements and penicillin/streptomycin (FGS, P/S solution, ScienCell), in accordance with the manufacturer’s protocol. The HTM cells were cultured at the bottom of poly-L-lysine-coated flasks (2 mg/cm\(^2\)).

**Shear Stress Experiments** As in previous shear stress experiments\(^\text{[15]}\), shear stress was applied to confluent HTM cells using a parallel plate-type flow chamber. Briefly, a flow chamber, a peristaltic pump (SJ1220, ATTO, Tokyo, Japan), and a medium reservoir compartment were all connected using silicone tubes in this flow circuit. The culture medium without serum was circulated continually with a peristaltic pump in a carbon dioxide (CO\(_2\)) incubator at 37°C with 5% CO\(_2\). In the current study, the HTM cells were cultured on glass plates and exposed to laminar shear stresses of the following magnitudes: 0 (static control, no fluid flow stimulation), 0.2, and 1.0 dyne/cm\(^2\).

The static cells were placed in an OmniTray (Thermo Fisher Scientific, Waltham, MA, USA) and cultured in the same amount of perfused medium. The shear stress within the flow chamber was calculated by the formula, \(\tau = \mu \frac{6Q}{a b}\), where \(\tau\) is the shear stress (dyne/cm\(^2\)), \(\mu\) the viscosity of the perfused fluid (0.00796 poise), \(Q\) the volumetric flow rate (ml/s), \(a\) the flow channel height (0.04 cm), and \(b\) the flow channel width (5.5 cm) in the cross section. We used Dulbecco’s Modified Eagle’s medium (Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 100 U/mL penicillin and 100 mg/mL streptomycin sulfate and GlutaMAX-I supplement (Life Technologies, Carlsbad, CA, USA) without serum to generate shear stress in a perfused medium. The perfused medium viscosities were measured using a viscometer (ViscoLab 4000, Japan Controls, Tokyo, Japan). The HTM cells were exposed to fluid flow for 12h, after which the cells cultured on glass plates were removed from the flow chamber and washed with phosphate buffered saline (PBS), and the collected HTM cells were used for the experiment.

**Phagocytosis Assay** HTM cells were plated into 48-well plates coated with poly-L-lysine. pHrodo green *Escherichia coli* bioparticles (Life Technologies) were suspended in a live cell imaging solution (Life Technologies) to a concentration of 1 mg/mL. The HTM cells were incubated at 37°C with pHrodo green bioparticles for 3h. The plates then were rinsed three times in live cell imaging solution and the HTM cells fixed for 10min at room temperature (RT) with 4% paraformaldehyde (Nacalai Tesque, Kyoto, Japan) and stained by Hoechst 33258 (Cosmo Bio, Tokyo, Japan) for 30min at RT. The HTM cells were photographed by a fluorescence microscope (BZ-X800, Keyence, Osaka, Japan).

**FN and COL Protein Expression in Response to TGF-β2** To investigate the FN and COL IV synthesis by HTM cells in response to TGF-β2 or Y-27632, a specific ROCK inhibitor, we conducted another experiment. The HTM cells were cultured into 10-cm plates and grown to a confluent state. The HTM cells with or without 10 μmol/L Y-27632 pretreatment for 30min were stimulated with TGF-β2 for 24h without washout of Y-27632. TGF-β2 was dissolved in 4 mmol/L hydrochloric acid, and the final concentration of hydrochloric acid was 200 μmol/L in culture medium. The cell lysates were collected and FN and COL IV proteins were quantified using Western blot analysis.
**Western Blot Analysis** The HTM cells were rinsed with PBS and lysed with RIPA lysis buffer (Merck Millipore, Germany), which contains phosphatase inhibitor cocktail tablets (Roche), protease inhibitor cocktail tablets (Roche, Basel, Switzerland), and phenylmethylsulfonyl fluoride. After that, the lysates were centrifuged at 13,000 rpm for 20 min at 4°C. The total protein concentration was measured by a NanoDrop Fluorospectrometer (Thermo Fisher Scientific). Equal quantities of protein were loaded into each well and separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis, and electroblotted onto nitrocellulose membranes. The membranes were blocked overnight at 4°C using PVDF blocking reagent (Toyobo, Osaka, Japan), then incubated for 1 h with Can Get Signal (Toyobo) containing the primary antibodies and horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG secondary antibody (#7076 and #7074, 1:10,000; Cell Signaling Technology). The membranes were incubated at RT with ECL Prime Western Blotting Detection Reagent (GE Healthcare, Piscataway, NJ, USA) and detected by an LAS-3000 Imager (Fujifilm, Tokyo, Japan). The data were analyzed using GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA). Quantitative data were analyzed using the Dunn’s nonparametric comparison for post-hoc testing following the Kruskal-Wallis test. The Mann-Whitney U test was used to analyze comparisons between two groups. *P*<0.05 considered to be statistically significant. All experiment was performed with the same lot of cell lines.

**Statistical Analysis** The data were analyzed using GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA). Quantitative data were analyzed using the Dunn’s nonparametric comparison for post-hoc testing following the Kruskal-Wallis test. The Mann-Whitney U test was used to analyze comparisons between two groups. *P*<0.05 considered to be statistically significant. All experiment was performed with the same lot of cell lines.

**RESULTS**

**Validation of the HTM Cell Phenotype** To be sure that the HTM cells from lot number 3423 expressed the TM cell phenotype, we investigated whether they expressed MYOC in response to DEX, FN and COL IV in response to TGF-β2 and presented phagocytic properties. MYOC induction in response to DEX is the most commonly used marker for TM cell phenotype[16]. TM cells have phagocytic properties, which maintain the aqueous outflow pathway[17]. The HTM cells used in the current study had significantly increased MYOC expression (1.6-fold, *P*<0.05; Figure 1A) induced by DEX (Figure 1A) and exhibited a phagocytotic function (Figure 1B). Further, the results of Western blot analysis for TGF-β2-induced FN proteins in cell lysate were significantly higher than in the control (1.9-fold, *P*<0.05; Figure 2A). Although COL IV proteins also increased with TGF-β2 treatment, the differences were not significant (2.15-fold, *P*=0.53; Figure 2B). The FN and COL IV protein levels did not differ significantly from the control when cells were treated with TGF-β2 and Y-27632 concomitantly (1.59-fold and 1.87-fold, respectively).

**Gene Expression of HTM Cells in Response to Shear Stress**

The HTM cells were exposed to shear stress collected with a scraper, and the total RNA was isolated by a NucleoSpin RNA kit (Takara, Shiga, Japan). The total RNA (25 mg/mL) underwent reverse transcription using a Transcriptor First Strand cDNA Synthesis Kit (Roche), in accordance with the manufacturer’s instructions, after which real-time PCR was subjected using the Universal ProbeLibrary and Light Cycler 480 (Roche). Specific primer pairs are listed in Table 1. The cycling conditions for all of the amplifications were as follows: an initial denaturation period for 5 min at 95°C, followed by 45 cycles of 10 s at 95°C, 30 s at 60°C, and 1 s at 72°C. The measurement of each gene expression signal was normalized to the glucose-6-phosphate dehydrogenase (G6PDH) gene signal. The relative fold changes in individual gene expression were determined using the 2^(-△△Ct) method.

**Table 1 Primary sequences for the analysis of gene expression by RT-PCR**

<table>
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<tr>
<th>Gene</th>
<th>Accession number</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<td>NM_000402</td>
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<td>TGCATTCAACACCTTGAC</td>
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</table>

**Mechanical fluid flow stimulation of HTM cells**

*COL1A2*: Collagen type 1 alpha 2; *COL4A2*: Collagen type 4 alpha 2; *COL6A1*: Collagen type 6 alpha 1; *FN1*: Fibronectin 1; *MMP-2*: Matrix metalloproteinase-2; *TIMP-2*: The tissue inhibitors of metalloproteinases-2; *PAI-1*: The plasminogen activator inhibitor; *G6PDH*: Glucose-6-phosphate dehydrogenase.
control, the difference was not significant (P=0.16; Figure 3). The MMP2 mRNA was significantly higher (0.2 dyne/cm², 2.09-fold vs static control, P<0.05 and 1.0 dyne/cm², 2.39-fold vs static control, P<0.05; Figure 4A), while the differences in the TIMP2 mRNA was not significant (0.2 dyne/cm², 1.22-fold vs static control, and 1.0 dyne/cm², 1.25-fold vs static control, P=0.98; Figure 4B). The plasminogen activator inhibitor-1 (PAI-1) mRNA was significantly higher (1.0 dyne/cm², 15.49-fold vs static control, P<0.01; Figure 4C).

**Phosphorylation of Smad2 in Response to Shear Stress**

TGF-β is the fibrogenic cytokine in TM cells, enhancing ECM and PAI-1 gene expression[18]. The canonical TGF-β2 signal transduction is mediated through phosphorylation of Smad2 protein. We examined the effect of shear stress on the phosphorylation of Smad2 in HTM cells. The phosphorylation of Smad2 was significantly higher in HTM cells exposed to shear stress after 12h than in the static control (1.0 dyne/cm², 10.79-fold vs static control, P<0.05; Figure 5).

**DISCUSSION**

The IOP is regulated by the quantitative balance between the secretion and drainage of aqueous humor; when the aqueous outflow decreases, the pressure increases and it is the main risk factor for primary openangle glaucoma[19]. There is evidence that progressive accumulation of misfolded proteins and malfunctioning of TM cells may may the aqueous outflow decreases[20]. In the current study, different intensities of fluid shear stress were applied to HTM cells to investigate the changes in gene and protein expression related to ECM. Shear stress induced phosphorylation of Smad2 (Figure 5)
and promotes COL4A2, COL6A1, FN1, and PAI-1 mRNA (Figures 3, 4). A previous report that the ECM gene, MMP-2, and PAI-1 gene expression were induced by TGF-β signaling [21]; therefore, HTM cells were thought to promote ECM gene up-regulation in the presence of shear stress. However, we could not detect the TGF-β1 and TGF-β2 protein in perfused cell culture medium, because these proteins were below the detection limit of enzyme-linked immunosorbent assay.

Keller et al [22] reported that when the TM cells detected the increased IOP, they released numerous activated proteinases, such as gelatinase A, which degraded the existing ECM and concurrently synthetized new proteins to replace the degraded matrix. Considering that flow stimulation corresponds to increased IOP in this study, it is considered that an increase in MMP leads to degradation of the ECM. Furthermore, considering the results of Bradley et al [8], i.e., that mechanical stretching of the TM cells up-regulates the MMP-2, we suggested that HTM cells under the mechanical fluid flow stimulation contributes to ECM turnover. In addition, clinically, the current results implied that the aqueous humor outflow through the TM may be necessary to maintain the physiologic IOP. According to Dan et al [23], the PAI-1 concentration in the aqueous humor contributes to the development of glaucoma, and we considered that shear stress in the TM may in part elevate the IOP.

In the current study, although the HTM cells were stimulated by steady fluid flow using our shear stress experimental system, it is presumed that the fluid flow in the TM of a normal eye is probably not a laminar or steady flow. A previous report that simulated the wall shear stress in each component of the conventional outflow pathway reported that the shear stress on the inner wall of SC was about 0.01 dyne/cm² [24]. Sherwood et al [25] reported that the normal physiologic value of the TM/SC resistance is 0.4 dyne/cm². Therefore, in the JCT, which is the primary tissue of the ECM metabolism in the TM, the shear stress might be equal to or less than that in the inner wall of SC. Considering these facts, our experiment should have been performed under weaker stress to approximate the reaction in vivo; however, we could not create shear stress that was less than 0.2 dyne/cm² due to the limitation of our experimental system. Furthermore, the TM tissue pulsates in conjunction with the heart rate [26]. Taken together, considering this histologic feature of the meshwork, the aqueous outflow in the TM may be turbulent. Further examination is necessary to clarify if the results of the current shear stress experiment also occur in vivo.

In conclusion, gene expression changes related to the ECM/ECM remodeling were observed as a result of continuous fluid shear stress in HTM cells in vitro, and up-regulation of FN and COL IV mRNA may be mediated by the phosphorylation of Smad2.

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Conflicts of Interest: Yoshida K, None; Kawai M, None; Utsunomiya T, None; Ishibazawa A, None; Song Y, None; Udo MSB, None; Tasaki Y, None; Yoshida A, None.
REFERENCES


