学位論文

Transforming Growth Factor-β Signaling Cascade Induced by Mechanical Stimulation of Fluid Shear Stress in Cultured Corneal Epithelial Cells.

（培養角膜上皮細胞における流体機械的刺激による Transforming Growth Factor-β のシグナル誘導に関する研究）

旭川医科大学大学院医学系研究科博士課程医学専攻

宇都宮　嗣了

（石羽澤明弘，長岡泰司，花田一臣，横田陽匡，石居信人，吉田晃敏）

Transforming Growth Factor-β Signaling Cascade Induced by Mechanical Stimulation of Fluid Shear Stress in Cultured Corneal Epithelial Cells

Tsugiaki Utsunomiya,[1] Akihiro Ishibazawa,[1] Taiji Nagaoka,[1] Kazuomi Hanada,[2] Harumasa Yokota,[1] Nobuhito Ishii,[1] and Akitoshi Yoshida[1]

[1]Department of Ophthalmology, Asahikawa Medical University, Asahikawa, Hokkaido, Japan
[2]Department of Medicine and Engineering Combined Research Institute, Asahikawa Medical University, Asahikawa, Hokkaido, Japan

Correspondence: Tsugiaki Utsunomiya, Department of Ophthalmology, Asahikawa Medical University, 2-1-1 Midorigaoka Higashi, Asahikawa, Hokkaido, 078-8510, Japan; utsunomy@asahikawa-med.ac.jp.

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Purpose. Because blinking is regarded as mechanical stimulation of fluid shear stress on the corneal epithelial cells, we investigated the effects of fluid shear stress on cultured human corneal epithelial cells (HCECs).

Methods. The HCECs were exposed to shear stress (0, 1.2, 12 dyne/cm^2) with the parallel-plate type of flow chamber. Wound healing, cellular proliferation, growth factor expression, TGF-β1 concentration in the culture supernatant, and phosphorylation of SMAD2 were investigated.

Results. Monolayers of HCECs exposed to shear stress had delayed wound healing and decreased proliferation compared with those of the static control (0 dyne/cm^2). With increasing shear stress, TGF-β1 expression and phosphorylation of SMAD2 increased significantly, but the levels of total TGF-β1 in the culture supernatant decreased significantly. Delayed wound healing, decreased proliferation, and phosphorylation of the SMAD2 by shear stress were canceled out with a TGF-β receptor inhibitor.

Conclusions. Fluid shear stress on the HCECs affected TGF-β signaling, which was associated with delayed wound healing. Mechanical stress by blinking might involve TGF-β signaling, and activation of TGF-β might be a key factor in wound healing of the corneal epithelium. Further studies should investigate the molecular mechanism of shear stress–induced activation of TGF-β.

Keywords: corneal epithelial cells, shear stress, transforming growth factor beta

Blinking plays an essential role in the distribution of tear fluid over the ocular surface. However, blinking can mechanically stimulate ocular surface cells. Cher[1] proposed the possibility of blink-related trauma in ocular surface disorders arising from mechanical friction or lubrication disorders of the eyes. Mechanical stress caused by blinking can vary in ocular-surface disorders, such as dry eye, superior limbic keratoconjunctivitis,[1] and lid-wiper epitheliopathy,[2,3] and cause corneal epithelial damage. Mechanical stress also can induce some effects on signaling cascades in the ocular surface cells. However, the details of these effects have not been determined.

Regarding the ocular tissues, mechanical shear stress resulting from fluid flow affects gene expression and signaling cascades, such as in retinal microvascular endothelial cells,[4,5] Schlemm's canal cells,[6] and corneal endothelial cells.[7] Blinking can be regarded as mechanical stimulation of fluid shear stress on the corneal epithelial cells, because blinking can induce movement of the tear fluid covering the corneal surface (Fig. 1). In fact, Carracedo et al.[8] showed that increased diadenosine polyphosphate in tears was induced by forced blinking in an in vivo experiment. Those authors also showed that shear stress stimulation with three rinses (aspiration and expulsion) induced diadenosine polyphosphate release from human corneal epithelium in an in vitro experiment.[8] However, the shear stress in their experiment was not quantitative. We investigated the effects of fluid shear stress on cultured human corneal epithelial cells (HCECs) using a flow chamber, which exposed the cells to variable levels of laminar shear stress.[4,9]

Methods

Cellular Culture

Human corneal epithelial cells (RCB2280; Riken Cell Bank, Ibaraki, Japan)[10] were cultured at the bottom of flasks containing Dulbecco's modified Eagle's Medium/Ham's F-12 (Nacalai Tesque, Kyoto, Japan) supplemented with 5% fetal bovine serum, 0.5% dimethyl sulfoxide, 5 μg/mL of insulin, 10 ng/mL of human epidermal growth factor (EGF), 100 units/mL of penicillin, and 100 μg/mL of streptomycin in an incubator at 37°C with 5% carbon dioxide. The cultured cells were passed by trypsinization in a 0.05% trypsin-2 mM ethylenediaminetetraacetic acid solution. Finally, the cells were seeded on glass plates (70 × 100 × 1.3 mm; Matsunami Glass, Kishiwada, Japan), which were incubated with 1% gelatin for 30 minutes. This study was performed in accordance with the tenets of the Declaration of Helsinki.

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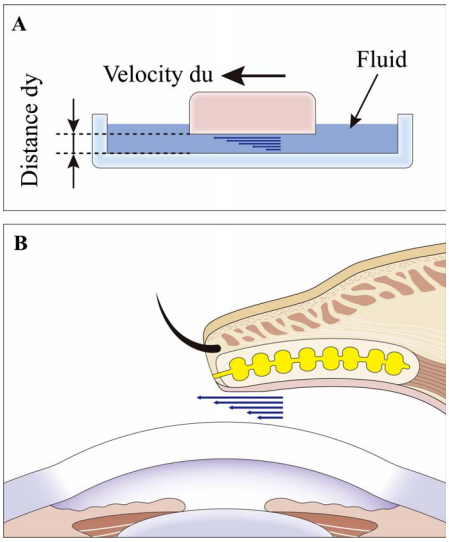

FIGURE 1. The schema of shear stress. (**A**) Objects in motion induce shear stress across fluid. Shear stress is defined by the following formula: shear stress = viscosity of fluid × velocity gradient, where the velocity gradient = velocity/distance. (**B**) Blinking induces shear stress on the corneal surface across the tear fluid. Shear stress on the corneal epithelial cells while blinking = viscosity of fluid × velocity of blinking/thickness of tear film.

Shear Stress Experiments

The confluent monolayers of the HCECs on glass plates were scratched using a blue pipette tip to produce horizontal wounds exposed to flow. After wounding, they were put in a parallel-plate type of flow chamber.[4,9] One side of the flow chamber consisted of a glass plate on which the HCECs were cultured; the other side was a polycarbonate plate. These two flat surfaces were held 0.02 cm apart by a polytetrafluoroethylene gasket. The components of the closed circuit, which included the flow chamber, a peristaltic pump (SJ1220; ATTO, Tokyo, Japan), and a medium reservoir, were connected using silicone tubes. Based on the structure of the flow chamber, the shear stress (τ, dyne/cm^2) acting on the cells was calculated using the following formula: $\tau = \mu \bullet 6Q/a^2b$, where μ is the viscosity of the perfused fluid (poise), Q the volumetric flow rate (mL/s), and a and b the height and width, respectively, of the channel in the cross section (cm).[4,9] Almost all HCECs (excluding only a small portion of the cells at the edge of the glass plate) were exposed to the laminar shear stress. To generate shear stresses using a medium, we used the above mentioned medium with or without 1 μM of SB431542 (Cellagen Technology, San Diego, CA, USA), which is a TGF-β receptor inhibitor. The medium viscosity was 0.00769 poise; shear rates of 0, 156, and 1560 1/s were applied to generate shear stresses of 0, 1.2, and 12 dyne/cm^2, respectively. To ensure the velocity of the fluid flow through the chamber, the flow volume was monitored using an ultrasonic transit time flow sensor (T106; Transonic Systems, Inc., Ithaca, NY, USA). These experiments were performed in an incubator at 37°C with 5% carbon dioxide. After exposure to the flow for 24 hours, the glass plates on which the HCECs were cultured were removed from the flow chamber and rinsed in PBS. The HCECs were collected and examined.

Wound Healing Assay

The images were captured by light microscopy (CKX41; Olympus, Tokyo, Japan) with the glass plates in the flow chamber. The wound areas in the images were calculated using Photoshop CS6 software (Adobe Systems, San Jose, CA, USA). The wound healing rate was calculated by dividing the area of healing by the area of the primary wounds.

Proliferation Assay

The proliferation assay was measured using a BrdU Labeling & Detection Kit (Roche, Basel, Switzerland), according to the manufacturer's instructions. The medium was supplemented with 10 μM of BrdU 2 hours before collection. The samples were fixed with 70% ethanol containing 50 mM of glycine, incubated with anti-BrdU mouse monoclonal antibody, and then incubated with anti-mouse fluorescein-labeled antibody. The samples were captured by confocal microscopy. The images were captured at three different sites around the center of the glass plate. The BrdU-positive cells were counted and the average was analyzed statistically.

Real-Time Reverse Transcription-Polymerase Chain Reaction Analysis

After exposure to shear stress, the HCECs were washed with PBS and collected with a scraper, and the total RNA samples were isolated from the cells (TRI Reagent, T9424; Sigma-Aldrich Corp., St. Louis, MO, USA). Reverse transcription (RT) was performed using a 20-μL mixture containing 1 μg of total RNA (Transcriptor First Strand cDNA Synthesis Kit; Roche), according to the manufacturer's instructions. After RT, real-time PCR was performed (Universal ProbeLibrary and LightCycler480; Roche). The specific primer pairs are shown in the Table. For all amplifications, the cycling conditions were as follows: an initial denaturation period for 5 minutes at 95°C,

TABLE. The Specific Primer Pairs

Gene	Forward Primers	Reverse Primers
TGFβ1	ACTACTACGCCAAGGAGGTCAC	TGCTTGAACTTGTCATAGATTTCG
TGFβ2	CCAAAGGGTACAATGCCAAC	CAGATGCTTCTGGATTTATGGTATT
TGFβ3	AAGAAGCGGGCTTTGGAC	CGCACACAGCAGTTCTCC
TGFβR2	TGGTGCTCTGGGAAATGAC	CACCTTGGAACCAAATGGAG
EGF	TGGTTGTGGTTCATCCATTG	TCACAGCCTCCGTTTTGATA
EGFR	GCCTTGACTGAGGACAGCA	TTTGGGAACGGACTGGTTTA
IGF	AAGGGACCCAAGAGATGAAGA	CCTTTGATTGCCACAATTCC
FGF7	CTGGCATGCAAGTGTGAGAC	CGAATGGTCACCCGAGTTT
PDGFB	CCTGGCTGTCCTTATCATCAC	GGCACCACTCACAGTGTTTTC
TGFα	CCTGGCTGTCCTTATCATCAC	GGCACCACTCACAGTGTTTTC
HGF	GATTGGATCAGGACCATGTGA	CCATTCTCATTTTATGTTGCTCA
GAPDH	CCCCGGTTTCTATAAATTGAGC	CACCTTCCCCATGGTGTCT

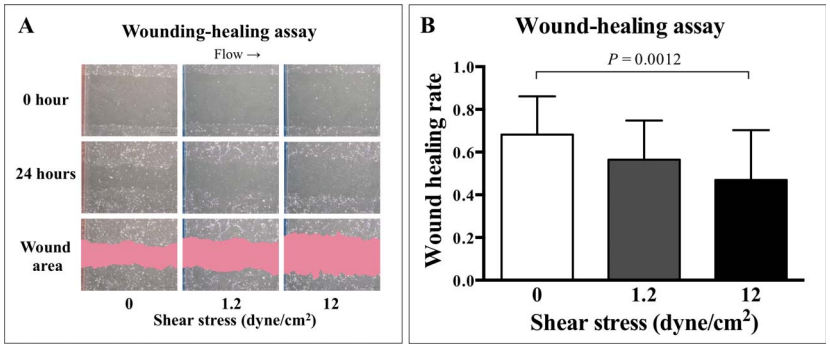

Figure 2. The relationship between the wound healing rate and shear stress. (**A**) Representative images of the wound healing assay using Photoshop CS6 software. (**B**) The wound healing rate of a horizontal wound exposed to flow. The wound healing rate at 12 dyne/cm^2 is decreased significantly compared with the static control (0 dyne/cm^2). The data are expressed as the means ± SDs (0 dyne/cm^2, $n = 29$; 1.2 dyne/cm^2, $n = 20$; 12 dyne/cm^2, $n = 20$).

followed by 45 cycles of 10 seconds at 95°C, 30 seconds at 60°C, and 1 second at 72°C. The quantification of each gene expression signal was normalized with respect to the signal for the *glyceraldehyde-3-phosphate dehydrogenase* gene. The relative fold changes in the expression of each gene were determined using the $2^{-\Delta\Delta Ct}$ method.

Enzyme-Linked Immunosorbent Assay

Transforming growth factor-β1 in the culture medium supernatant was measured using an ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Culture medium supernatants were collected from the HCECs with or without exposure to shear stress for 24 hours. To activate latent TGF-β1 to immunoreactive TGF-β1 detectable by the kit, the samples were processed by acid activation and neutralization. The optical density of the samples and TGF-β1 standard were determined using a microplate reader.

Western Blotting

After exposure to shear stress, the HCECs were washed with PBS and lysed with RIPA Lysis Buffer (Merck Millipore, Darmstadt, Germany) containing protease inhibitor cocktail tablets (Roche) and phosphatase inhibitor cocktail tablets (Roche) and phenylmethylsulfonyl fluoride. The lysates were centrifuged and the resultant supernatants were collected. The protein concentrations were measured using a NanoDrop Fluorospectrometer (Thermo Fisher Scientific, Inc., Rockford, IL, USA). A total of 200 μg of protein was loaded per well and separated by electrophoresis with 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with PVDF Blocking Reagent (Toyobo, Osaka, Japan). The membranes were incubated in Can Get Signal (Toyobo) containing the following antibodies for 1 hour: SMAD2, phosphorylated SMAD2, β-actin (1:1000 dilution; Cell Signaling Technology, Danvers, MA, USA), and horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG secondary antibody (1:3000 dilution; Cell Signaling Technology). Restore Western Blot Stripping Buffer (Thermo Fisher Scientific, Waltham, MA, USA) was used for reprobing. The membranes were exposed to ECL Prime Western Blotting Detection Reagent (GE Healthcare, Piscataway, NJ, USA) and examined using LAS-3000 Imager (Fujifilm, Tokyo, Japan).

Statistical Analysis

All values are expressed as the mean ± SD. One-way ANOVA was used to compare the mean values. When a significant F ratio was observed, Tukey's multiple comparison tests was used to identify significant differences. Differences were considered significant for RT-PCR analysis at P less than 0.01 and for other analyses at P less than 0.05.

Results

Wound Healing

Representative images of the wound healing assay are shown in Figure 2A. Obvious changes in morphology and orientation were not observed by light microscopy (Supplementary Fig.).

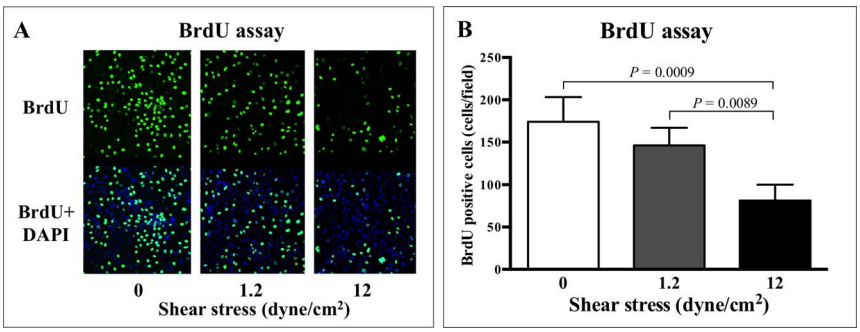

Figure 3. The relationships between cellular proliferation and shear stress. (**A**) Representative images of the BrdU assay: BrdU (*fluorescein*), DAPI (*blue*). (**B**) The number of BrdU-positive cells at 12 dyne/cm^2 is decreased significantly compared with the static control (0 dyne/cm^2) and low shear stress (1.2 dyne/cm^2). The data are expressed as the means ± SDs (0 dyne/cm^2, $n = 4$; 1.2 dyne/cm^2, $n = 4$; 12 dyne/cm^2, $n = 4$).

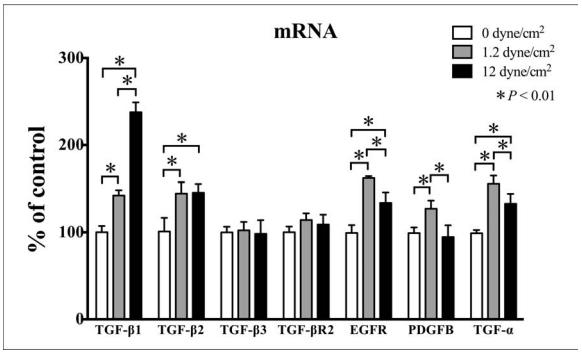

FIGURE 4. Gene expression after exposure to shear stress for 24 hours. The expression of TGF-β1 in cells exposed to shear stress is increased significantly compared with that in the static cells. The data are expressed as the means ± SDs (0 dyne/cm², $n = 7$; 1.2 dyne/cm², $n = 4$; 12 dyne/cm², $n = 4$).

The wound-healing rate of the horizontal wounds exposed to flow at 12 dyne/cm² decreased significantly compared to the static control at 0 dyne/cm² (Fig. 2B).

Cellular Proliferation

Representative images of the BrdU assay are shown in Figure 3A. The number of BrdU-positive cells decreased significantly at 12 dyne/cm² compared with the static control (0 dyne/cm²) and low shear stress (1.2 dyne/cm²; Fig. 3B).

Gene Expressions of Growth Factors

Figure 4 shows the gene expressions of the growth factors. Transforming growth factor-β1 expression in the cells exposed to flow increased significantly with increasing shear stress. The increased TGF-β1 expression was notable compared with those of TGF-β2, epidermal growth factor receptor (EGFR), platelet-derived growth factor (PDGFB), and TGF-α, which increased significantly. Real-time RT-PCR analysis did not detect expression of insulin-like growth factor, fibroblast growth factor 7, or hepatocyte growth factor. The expression of EGF was detected, although it was very low.

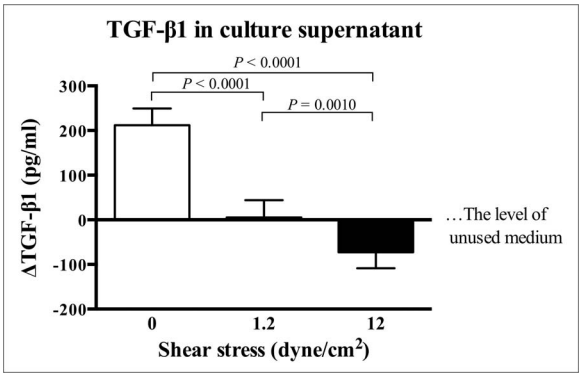

FIGURE 5. The levels of total TGF-β1 in the culture supernatant after exposure to shear stress for 24 hours. The levels of total TGF-β1 in the culture supernatant in a static control (0 dyne/cm²) are increased from baseline (unused medium), although those at 12 dyne/cm² are decreased from baseline. The levels of total TGF-β1 in the culture supernatant are decreased significantly with increasing shear stress. The data are expressed as the means ± SDs (0 dyne/cm², $n = 6$; 1.2 dyne/cm², $n = 4$; 12 dyne/cm², $n = 4$).

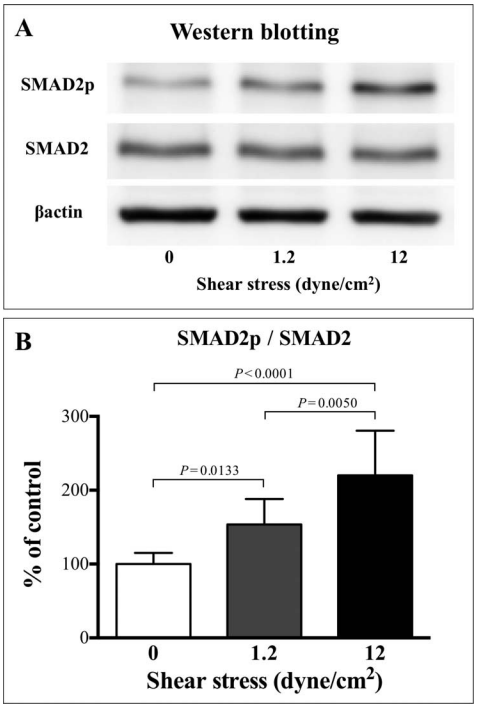

FIGURE 6. The phosphorylation of SMAD2 in cells exposed to shear stress. (**A**) Representative images of Western blotting. (**B**) The phosphorylation of SMAD2 in cells exposed to flow is increased significantly with increasing shear stress. The data are expressed as the means ± SDs (0 dyne/cm², $n = 12$; 1.2 dyne/cm², $n = 8$; 12 dyne/cm², $n = 8$).

Levels of Total TGF-β1 in the Culture Supernatant

The levels of total TGF-β1 (latent TGF-β1 and active TGF-β1) in the culture supernatant were measured by ELISA (Fig. 5). Those at the static control (0 dyne/cm²) increased from baseline (unused medium); however, those at 12 dyne/cm² decreased from baseline. The levels of total TGF-β1 in the culture supernatant decreased significantly with increasing shear stress.

Phosphorylation of SMAD2

The phosphorylation of SMAD2 in HCECs exposed to flow increased significantly with increasing shear stress (Fig. 6).

Experiments With the TGF-β Receptor Inhibitor

The results of Western blotting, the wound-healing assay, and the BrdU assay with SB431542, which is a TGF-β receptor inhibitor, are shown in Figure 7. The phosphorylation of SMAD2 in HCECs exposed to flow was canceled with SB431542 (Figs. 7A, 7B). Furthermore, HCECs exposed to flow with SB431542 did not have significantly delayed wound healing or decreased proliferation compared with those without SB431542 (Figs. 7C, 7D).

Discussion

Blinking can induce mechanical force on the corneal surface in the form of shear stress resulting from tear-fluid flow hydrodynamics. The motion of an object induces shear stress across fluid. Shear stress is defined by the following formula: shear stress = viscosity of fluid × velocity gradient, where the

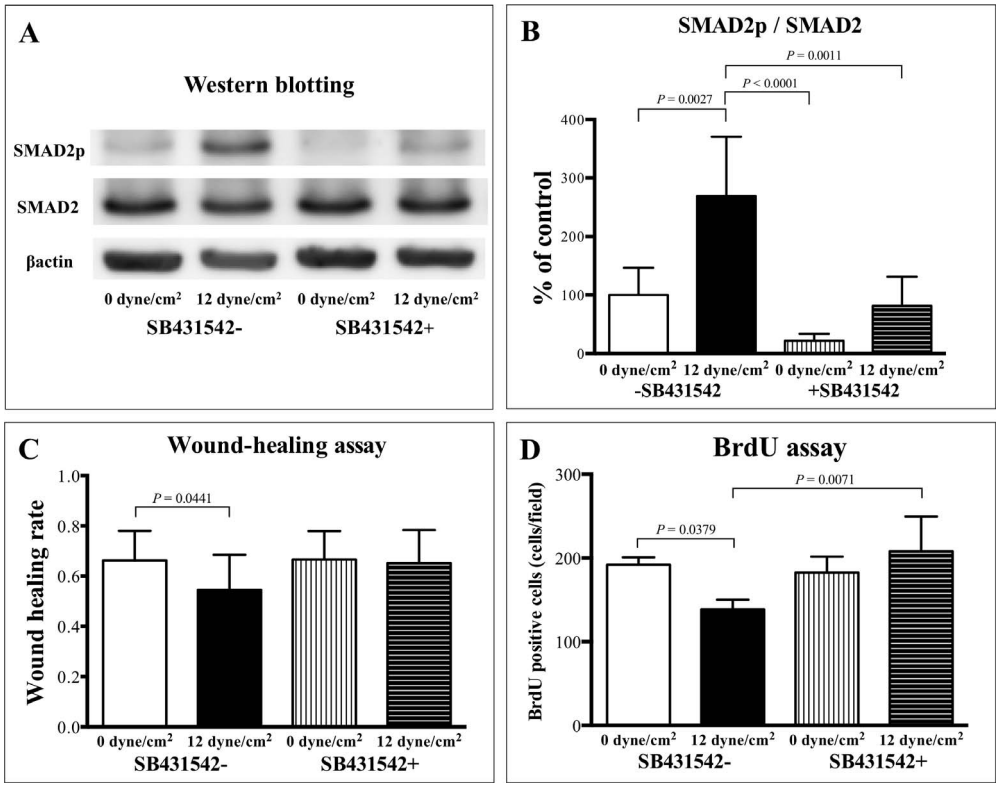

FIGURE 7. Results of Western blotting, the wound-healing assay, and the BrdU assay with SB431542, a TGF-β receptor inhibitor. The data are expressed as the means ± SDs. (**A**) Representative images of Western blotting. (**B**) The phosphorylation of SMAD2 in cells exposed to flow is canceled by SB431542 (SB431542- , 0 dyne/cm^2, $n = 5$; 12 dyne/cm^2, $n = 4$; SB431542+, 0 dyne/cm^2, $n = 5$; 12 dyne/cm^2, $n = 5$). (**C**) The delayed wound healing in cells exposed to flow is canceled by SB431542 ($n = 14$ for all). (**D**) The decreased proliferation in cells exposed to flow is canceled by SB431542 ($n = 4$ for all).

velocity gradient = velocity/distance (Fig. 1). Applying this formula to the ocular surface, shear stress on the corneal epithelial cells while blinking = viscosity of tear fluid × velocity of blinking/thickness of tear film (Fig. 1). This formula shows that shear stress on the corneal epithelial cells is inversely proportional to the tear film, suggesting that shear stress on the corneal epithelial cells might increase in patients with aqueous-deficient dry eye. In fact, the levels of diadenosine polyphosphate increased in patients with dry eye[11] and Sjögren's syndrome.[12] In the current study, we investigated the effects of fluid shear stress on cultured HCECs using a flow chamber, which facilitated stable exposure of the cells to quantitative shear stress.[4,9] We confirmed for the first time that fluid shear stress on the HCECs affected TGF-β signaling, which was associated with delayed wound healing.

Transforming growth factor-β is a multifunctional cytokine that regulates several cellular processes in the cornea.[13] Transforming growth factor-β1 is produced by the human lacrimal glands,[14] corneal epithelium, and conjunctival epithelium,[15] and can be detected in tears.[16] The levels of active TGF-β1 in tears[17] and the expressions of TGF-β1 in the conjunctiva[18–20] and minor salivary glands[21] were elevated in patients with dry eye. Moreover, TGF-β inhibits proliferation, migration, and adhesion of corneal epithelial cells via induced-EGF.[22–26] In the current study, shear stress induced delayed wound healing (Fig. 2) and decreased cellular proliferation (Fig. 3), both of which were canceled by the TGF-β receptor inhibitor (Fig. 7). Therefore, delayed wound healing and decreased cellular proliferation induced by shear stress might have resulted from the effect of TGF-β, which inhibits the effects of EGF.

Activation of TGF-β is crucial in TGF-β–regulated biologic activity.[13,27] Transforming growth factor-βs are secreted in a latent form, and most remain latent and cannot bind to its cellular surface receptors. By dissociation from latency-associated peptide, TGF-βs become active, bind to the TGF-β receptor, and induce signal transduction mainly via phosphorylation of SMAD. In the current study, shear stress increased the mRNA of TGF-β1 in HCECs (Fig. 4) and the phosphorylation of SMAD2 (Fig. 6) and decreased the levels of total TGF-β1 in the culture supernatant from baseline (unused medium; Fig. 5). These findings might indicate activation of TGF-β1 by which TGF-β can bind to the TGF-β receptor and induce phosphorylation of SMAD2. As a result, TGF-β1 in the culture supernatant was consumed and the expression of TGF-β1 increased by negative feedback. In signal transduction of TGF-β by shear stress, the mechanism of the activation of TGF-β might be a key factor. Various factors have been reported to activate the latent form of TGF-β[27] (i.e., plasmin, matrix metalloproteinase, thrombospondin, integrin, etc.). Further research is needed to elucidate the mechanism of activation of TGF-β by shear stress.

The current study had some limitations. First, although we focused on the role of TGF-β1 among the growth factors (i.e., TGF-β1, -β2, EGFR, PDGFB, and TGF-α) because RT-qPCR showed that TGF-β1 was greatly altered by shear stress (Fig. 4), we could not eliminate the possibility that other growth factors might have affected the findings in this study. Second, we could not create more than 12 dyne/cm^2 of shear stress due to the limitation of the peristaltic pump. Shear stress on the corneal epithelial cells during blinking cannot be calculated because the tear film thickness is unknown during blinking.

When calculated based on the thickness of the aqueous layer in the tear film when the eye is open,[28] the shear stress on the corneal epithelial cells might be higher than in this experimental condition. Nevertheless, this experimental condition affected TGF-β signaling possibly because of the amount of microvilli and membrane-associated mucins, which should cushion and protect the corneal epithelial cells from shear stress during blinking.[29,30] The corneal epithelial cells in vivo have microvilli covered with membrane-associated mucins; meanwhile, the corneal epithelial cells in vitro have fewer microvilli and membrane-associated mucins due to passage accompanied by trypsinization. Another in vivo study is needed. Third, we used SV40 immortalized HCECs,[10] which are the most often used HCECs, because numerous uniform cells were needed for shear stress experiments. The SV40 immortalized HCECs have altered genomic contents.[31] We need to interpret the results in the current study with caution. Fourth, our experimental system was a closed circuit that included just corneal epithelial cells. That might be why shear stress decreased the level of total TGF-β1 in the culture supernatant from baseline, although elevated levels of active TGF-β1 in the tears of patients with dry eye have been reported.[17] Corneal and conjunctival epithelial cells are both sources of TGF-β and a potential target for TGF-β, and conjunctival epithelial cells secreted TGF-β1 more than corneal epithelial cells.[15] In addition, conjunctival epithelial cells might have more effects than the cornea in in vivo experiments, because the conjunctival area is larger than that of the cornea on the ocular surface. Further investigations of the effects of shear stress on conjunctival epithelial cells and in vivo experiments on forced blinking are needed.

In conclusion, the current study showed that shear stress affects TGF-β signaling in cultured HCECs, suggesting that mechanical stress caused by blinking might affect TGF-β signaling, which is associated with wound healing.

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