

## Tranilast Inhibits TGF- $\beta$ 1 Secretion without Affecting its mRNA Levels in Conjunctival Cells

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**Key words:** Tranilast; TGF- $\beta$  1; secretion; Chang Conjunctiva cells; Filtration bleb; glaucoma filtration surgery

**Abbreviations:** TGF- $\beta$ , Transforming factor- $\beta$ ; RT-PCR, reverse transcriptase-polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; RNA, ribonucleic acid; mRNA, messengerRNA; 5-FU, 5-fluorouracil; MMC, mitomycin C

**The present study was conducted to assess the effect of Tranilast, a drug developed as anti-keeloid and anti-hypertrophic scar agent, on the level of transforming growth factor- $\beta$  1 (TGF- $\beta$  1) mRNA, and on TGF- $\beta$  1 secretion in Chang Conjunctiva cells. TGF- $\beta$  1 mRNA was not detected in Chang Conjunctiva cells by Northern blot analysis, but reverse transcriptase-polymerase chain reaction (RT-PCR) analysis confirmed the presence of TGF- $\beta$  1 mRNA. Tranilast, whereas the drug had no effect on the levels of TGF- $\beta$  1 mRNA and cellular protein, time- and dose-dependently inhibited TGF- $\beta$  1 secretion from Chang Conjunctiva cells in the enzyme-linked immunosorbent assay (ELISA) analysis. TGF- $\beta$  1 is suggested to cause fibroblast proliferation, that obstructs aqueous humor filtration route after glaucoma filtration surgery. Tranilast, potentially inhibiting TGF- $\beta$  1 secretion, therefore, could be a promising drug to prevent from scarring after glaucoma filtration surgery.**

Several lines of evidence have pointed to a significant role for cytokines in wound healing processes: Cytokines allow fibroblasts to migrate towards wound; the fibroblasts proliferate, cover the wound, and form cicatricial tissue by producing extracellular matrix like collagen; and then the wound eventually heals [1,2]. Transforming factor- $\beta$  (TGF- $\beta$ ) is a member of cytokine family, involving cell proliferation, migration, differentiation and apoptosis [3,4]. Until now, three isoforms of mammalian TGF- $\beta$  s, such as TGF- $\beta$  1, TGF- $\beta$  2, and TGF- $\beta$  3, with 70-80% homologous in the amino acid sequence between them, have been identified. While the TGF- $\beta$  s exhibit similar actions in the vitro studies, independent actions between the three isoforms are found in the in vivo study using knockout mice [5]. Interestingly, aqueous humor in the eye with primary open angle glaucoma contains high concentrations of TGF- $\beta$  2 [6]. It is shown that the conjunctival tissue produces TGF- $\beta$  [7], and that TGF- $\beta$  1 promotes proliferation of cultured human Tenon's capsule fibroblasts [8]. These raise the possibility that TGF- $\beta$  might be employed in the filtering bleb formation after glaucoma surgery. After glaucoma filtration surgery, it is extremely important to secure the aqueous humor filtration pathway to keep the attained intraocular pressure-lowering effect. However, it is not established a promising method to prevent aqueous humor filtration pathway from occluding in a wound healing process [1,2].

Tranilast, N-(3,4-dimethoxycinnamoyl) anthranilic acid, has been developed as a drug against allergy, keloid and hypertrophic scar, perhaps by inhibiting secretion of TGF- $\beta$  1 [9].

Then, we hypothesized that Tranilast might prevent conjunctival cicatrization after glaucoma surgery. The effect of Tranilast on secretion of TGF- $\beta$  1 from the eye conjunctiva, however, is unknown. To address this question, we assessed the effects of Tranilast on the level of TGF- $\beta$  1 mRNA, and on TGF- $\beta$  1 secretion from the adult human conjunctiva-derived cell strains, Chang Conjunctiva, by carrying out Northern blotting, reverse transcriptase-polymerase chain reaction (RT-PCR), and enzyme-linked immunosorbent assay (ELISA).

## **MATERIALS AND METHODS**

### *2.1. Cell culture*

Chang Conjunctiva cells (ATCC No. CCL-20.2, Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) were cultured in Medium 199, pH7.2-7.4 (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT, USA) under 95% air and 5% CO<sub>2</sub> at 37°C. Next day, medium was replaced with fresh medium containing 0, 30, or 300  $\mu$  M of Tranilast (Kissei Pharmaceutical Co., Ltd. Matsumoto, Japan).

### *2.2. Northern blotting and RT-PCR*

Eight and 24-h after adding Tranilast, total cellular RNAs were prepared from Chang Conjunctiva cells with the method of Acid Guanidinium Phenol Chloroform using ISOGEN reagent (Wako Pure Chemical Industries, Ltd., Osaka, Japan). RNA concentrations were determined by measuring ultra-violet (UV) absorption at 260 nm; the optical density of 1 mg/ml RNA should be 25. For Northern blotting, 20  $\mu$  g RNA per a lane was loaded onto a formaldehyde-denatured agarose gel. After electrophoresis, RNA was blotted on a Hybond N nylon membrane (Amersham International plc., Buckinghamshire, UK), and fixed by UV irradiation. The quality of RNA was confirmed by methylene blue-staining of RNA on the membrane (0.02% methylene blue in 0.3 M sodium acetate, pH5.2). Hybridization was performed successively with <sup>32</sup>P-labelled human TGF- $\beta$  1 oligonucleotide probe (Clontech Laboratories, Inc., Palo Alto, CA, USA) and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) complementary deoxyribonucleic acid (cDNA) probe (Clontech Laboratories, Inc., Palo Alto, CA, USA) for control. Hybridized image was analyzed in a BAS2000 II Image Analyzer system with the BASStation software (Fuji Photo Film Co., Ltd., Tokyo, Japan).

For RT-PCR of TGF- $\beta$  1 and G3PDH, 25  $\mu$  l of cDNAs were made from 5  $\mu$  g of all RNAs using with First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech Co., Ltd., Tokyo, Japan), and 1  $\mu$  l each was amplified with using thermocycler Gene Amp PCR System 9700 (Perkin-Elmer, Foster City, CA, USA) by 18-23 cycles for G3PDH mRNA or 23-28 cycles for TGF- $\beta$  1 mRNA at degeneration temperature 94°C for 30s, at annealing temperature 57°C for 30s, and at expansion temperature 68°C for 40s. The PCR product was confirmed by electrophoresing with 1.0% agarose gel (ethidium bromide mixture) and by taking a picture on the transilluminator.

### *2.3. ELISA*

On 8 and 24-h culture of Chang Conjunctiva cells in Medium 199 supplemented with 0.2mg/ml bovine serum albumin in the presence and absence of Tranilast, culture supernatant was collected and kept at frozen -80°C. TGF- $\beta$  1 in the supernatant was assayed using with Quantikine human TGF- $\beta$  1 ELISA Kit (R&D Systems, Minneapolis, NM, USA) according to a protocol described. Cells were lysed with a cytolytic reagent (Promega, Madison, WI, USA) and cellular protein was determined using with a BioRad protein assay DC reagent (BioRad Laboratories, Hercules, CA, USA).

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### 2.4. Analysis of cultured human mesangial cells

Human mesangial cells (Sankojunyaku, Tokyo, Japan) are well recognized to express TGF- $\beta$  1 [10,11]. Human mesangial cells, therefore, were cultured in an MSBM low-serum medium (Sankojunyaku, Tokyo, Japan), as positive control for TGF- $\beta$  1 detection. After three days, medium was replaced with fresh MSBM medium containing 0, 30, or 300  $\mu$  M of Tranilast. Twenty four hours later, TGF- $\beta$  1 expression was analysed with Northern blotting and RT-PCR, and TGF- $\beta$  1 secreted in the culture medium was determined by ELISA.

### 2.5. Data analysis

Data were analyzed on a microcomputer and statistical significance was evaluated with the method of Bonferroni programmed by Kaneko (Kaneko S, Macintosh handbook. pp127-129, Yodosha, Tokyo, 1992).

## RESULTS

### 3.1. Northern blot analysis

RNAs from Chang Conjunctiva cells and mesangial cells were well hybridized with G3PDH probe (Fig. 1A, 1C), indicating good quality of RNA preparations tested here. RNAs from Chang Conjunctiva cells exhibited no hybridization with TGF- $\beta$  1 probe still after 22-h exposure on the imaging plate (Fig. 1B). In contrast, clear hybridization with TGF- $\beta$  1 probe was found in RNAs from mesangial cells, even though the RNAs loaded was lesser than half of RNAs from Chang Conjunctiva cells (Fig. 1D). Quantitative study of Northern blotting was carried out in RNAs from human mesangial cells and the intensity of TGF- $\beta$  1-specific band was normalized with that of G3PDH band (Table I). The result indicates that Tranilast has no effect on the level of TGF- $\beta$  1 mRNA in human mesangial cells.

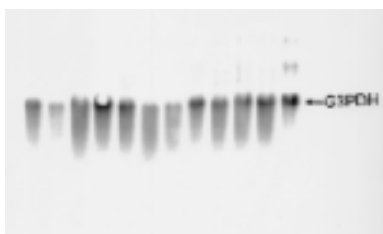


Fig. 1A.

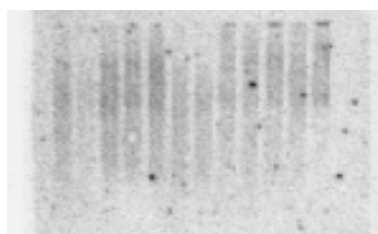


Fig. 1B.

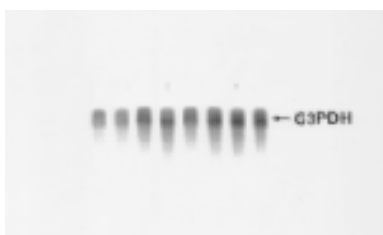


Fig. 1C.

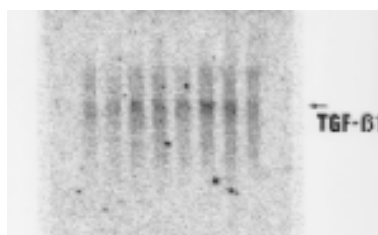


Fig. 1D.

### Fig. 1. Northern blot analysis.

Chang Conjunctiva cells were treated with Tranilast at 0, 30, 300  $\mu$  M for 8 h and at 0, 30, 300  $\mu$  M for 24 h and RNA preparations from cells were hybridized with G3PDH probe (A) and TGF- $\beta$  1 probe (B) (1-h exposure on the BAS2000 imaging plate) for each two lanes from the left, respectively. Human mesangial cells were treated with Tranilast at 0, 30, 300  $\mu$  M for 8 h and at 0, 30, 300  $\mu$  M for 24 h and RNA preparations from cells were hybridized with G3PDH probe (C) and TGF- $\beta$  1 probe (D) (1-h exposure on the BAS2000 imaging plate) for each two lanes from the left, respectively.

Table 1. Quantitative determination by Northern blotting for mesangial cells

Lane	Treated with Tranilast	TGF- $\beta$ 1 (psi/A)	G3PDH (psi/A)	100xTGF- $\beta$ 1/G3PDH
1	DMF only	80.68	6920	1.17
2	DMF only	87.78	7114	1.23
3	30 $\mu$ M	124.62	11192	1.11
4	30 $\mu$ M	117.10	10734	1.09
5	300 $\mu$ M	117.94	10453	1.13
6	300 $\mu$ M	155.55	13180	1.18
7	Not added	153.53	13614	1.13
8	Not added	105.11	12511	0.84

### 3.2. RT-PCR analysis

TGF- $\beta$  1 mRNA from Chang Conjunctiva cells was amplified with an RT-PCR technique. TGF- $\beta$  1 mRNA was detectable in Chang Conjunctiva cells, but there was no obvious difference in the level of TGF- $\beta$  1 mRNAs between Tranilast-treated cells and -untreated cells, independently the number of cycles (Fig. 2A, 2B, 2C), suggesting that the drug has no effect on the expression of TGF- $\beta$  1. Likewise, the level of G3PDH mRNAs was not also affected by Tranilast (Fig. 2D, 2E, 2F).

### 3.3. Quantitative analysis by ELISA

The amount of TGF- $\beta$  1 secreted from Chang Conjunctiva cells was determined with an ELISA technique. TGF- $\beta$  1 secreted from untreated cells was  $49.4 \pm 1.9$  and  $120.0 \pm 12.4$  pg/well at 8 and 24 h, respectively (control) (Fig. 3). 300  $\mu$  M Tranilast significantly inhibited TGF- $\beta$  1 secretion to  $65.8 \pm 10.7\%$  of control levels at 8-h treatment ( $P < 0.01$ ), but no significant inhibition was found with 30  $\mu$  M Tranilast (Fig. 3). More prominent effect was obtained with 24-h treatment with Tranilast; the drug significantly inhibited TGF- $\beta$  1 secretion in a dose-dependent manner ( $72.8 \pm 9.6\%$  of control levels for 30  $\mu$  M,  $P < 0.01$ ; and  $23.0 \pm 2.5\%$  of control levels for 300  $\mu$  M,  $P < 0.01$ ) (Fig. 3). These, taken together with the fact that there was no significant difference in the level of cellular protein (Fig. 3), suggest that Tranilast inhibits TGF- $\beta$  1 secretion from cells.

Much larger amount of TGF- $\beta$  1 ( $939.5 \pm 15.5$ pg/well) was secreted from human mesangial cells (Fig. 4), reaching approximately 10 times of the secretion from Chang Conjunctiva cells, Tranilast significantly inhibited TGF- $\beta$  1 secretion in a dose-dependent manner at 24-h treatment ( $84.0 \pm 1.0\%$  of control levels for 30  $\mu$  M,  $P < 0.01$ ; and  $61.4 \pm 4.5\%$  of control levels for 300  $\mu$  M,  $P < 0.01$ ) (Fig. 4). In the microscopic study, no change in the morphological features and the cell density was found with 24-h treatment with 300  $\mu$  M Tranilast (data not shown), further supporting the evidence that Tranilast is implicated in the regulation of TGF- $\beta$  1 secretion without affecting cell viability.

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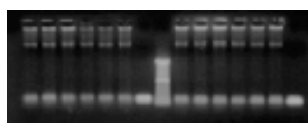
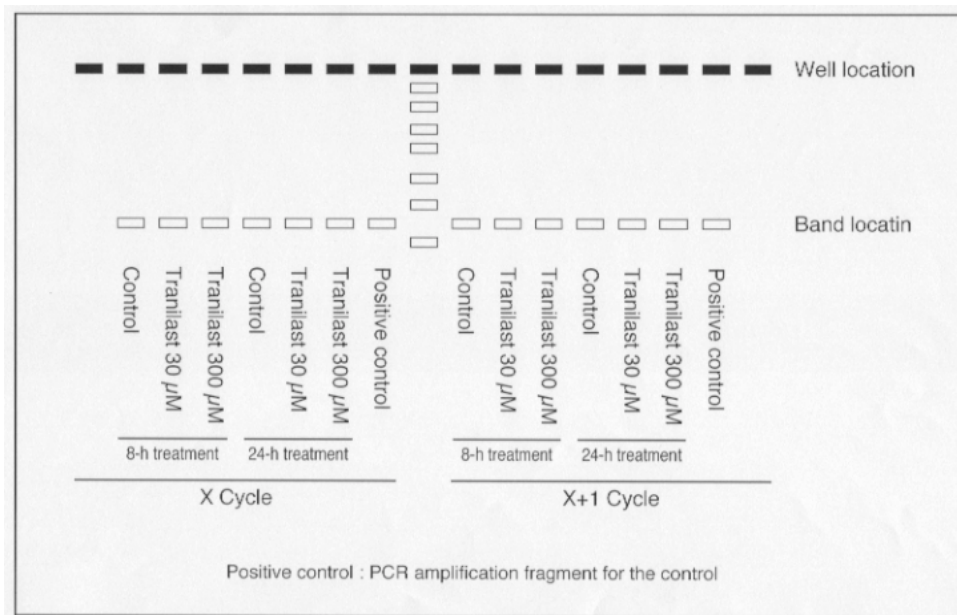


Fig. 2A.

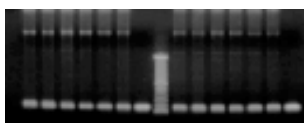


Fig. 2B.

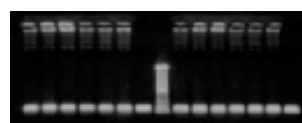


Fig. 2C.

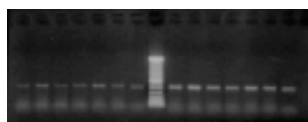


Fig. 2D.

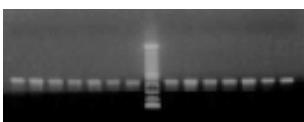


Fig. 2E.

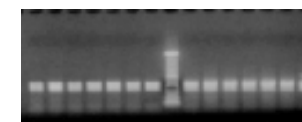


Fig. 2F.

**Fig. 2.** Agarose gel electrophoretic photograph of RT-PCR Product.

TGF- $\beta$  1 mRNA (A, B, C); 23, 24 cycles amplification (A), 25, 26 cycles amplification (B), 27, 28 cycles amplification (C). G3PDH (D, E, F); 18, 19 cycles amplification (D), 20, 21 cycles amplification (E), 22, 23 cycles amplification (F).

### DISCUSSION

In the present study, TGF- $\beta$  1 mRNAs were not detectable in Chang Conjunctiva cells by Northern blotting analysis, but detected by amplifying them with an RT-PCR technique. This would explain that TGF- $\beta$  1 mRNA is expressed in Chang Conjunctiva cells, but with the extent being considerably lesser. There was no obvious difference in the level of TGF- $\beta$  1 mRNAs between Tranilast-treated and -untreated Chang Conjunctiva cells, suggesting that Tranilast had little effect on expression of TGF- $\beta$  1 mRNA although a quantitative PCR method would be required to assess the precise difference. In the ELISA study, Tranilast significantly reduced the amount of TGF- $\beta$  1 in culture medium, in a dose- and treatment duration-dependent manner. A similar inhibition was found in human mesangial cells. Otherwise, Tranilast had no effect on the level of cellular protein and on morphological features at 24-h treatment. Taken together, it appears that Tranilast is not involved in

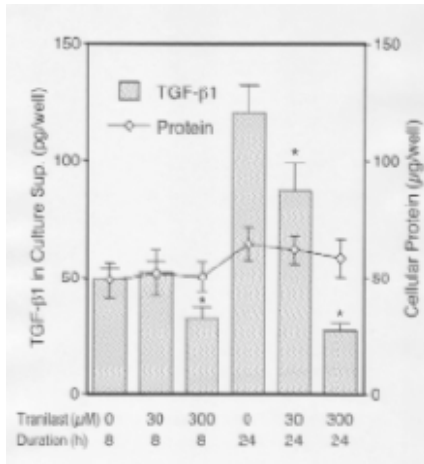


Fig. 3.

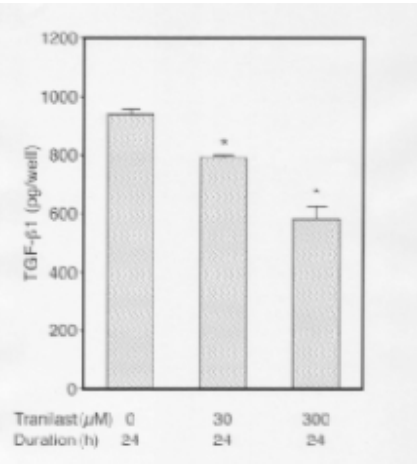


Fig. 4.

**Fig. 3.** Effect of Tranilast on cellular protein levels and TGF-β 1 secretion in Chang Conjunctiva cells.

Cells were treated with Tranilast at 0, 30, 300 μ M for 24 h, and cellular protein and secreted TGF- β 1 were assayed (N=6). \*P<0.01, Bonferroni test.

**Fig. 4.** Effect of Tranilast on TGF- β 1 secretion from human mesangial cells.

Cells were treated with Tranilast at 300 μ M for 24 h and secreted TGF- β 1 were assayed (N=3). \*P<0.01, Bonferroni test.

protein synthesis and cell death, but that instead, the agent is capable of inhibiting TGF- β 1 secretion from eye conjunctival cells. A variety of substances, such as corticosteroids, 5-FU, and MMC, are clinically used to keep the aqueous humor filtration route after glaucoma filtration surgery. Corticosteroids is shown to cause infection and raise intraocular pressure [10,11,12]. As far as 5-FU and MMC are concerned, usage at concentrations effective to keep the aqueous humor filtration route causes corneal damage, leak from conjunctival wound, and bleb rupture, namely complications [13]. The problem of these two drugs, 5-FU and MMC, is that the concentration at which the drug is efficacious is overlapped with a concentration at which adverse reactions develop. Thus, these two drugs also are not ideal anticatrizants. The action mechanism of Tranilast is different from these drugs and it is safe. Even if it is used with the above drugs together, it is considered to not inhibit effect for maintenance of the aqueous humor filtration route. So far as our knowledge is concerned, there is no serious side effect by using eye drops [14].

Lines of evidence suggest that TGF- β is secreted in aqueous humor from conjunctival cells, promoting fibroblast proliferation, and then leading to filtering bleb formation after glaucoma surgery [6,7,8]. In the present study, Tranilast inhibited TGF- β 1 secretion from Chang Conjunctiva cells. Tranilast, thus, could be a promising drug to prevent from scarring after glaucoma filtration surgery.

In conclusion, the results of the present study suggest that Tranilast inhibited TGF- β 1 secretion from the eye conjunctiva without affecting its expression.

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