ELSEVIER

Effects of Tranilast on Cultured Rabbit Corneal Keratocytes and Corneal Haze After Photorefractive Keratectomy

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Purpose: In vitro and in vivo studies were performed to elucidate the effects of tranilast on cellular proliferation and collagen synthesis.

Methods: Subculturing was carried out using keratocytes from rabbits that underwent photorefractive keratectomy (PRK) and developed corneal haze, and keratocytes from normal rabbit cornea.

Results: Tranilast suppressed proliferation in cultured keratocytes from the corneal haze region at doses of 30 and 300 μmol/L and collagen synthesis at doses of 3, 30, and 300 μmol/L. Normal corneal cultures showed suppression of keratocyte proliferation and collagen synthesis only at a high dose of tranilast (300 μmol/L). Betamethasone suppressed proliferation of keratocytes in both haze and normal cornea at a dose of 10 μmol/L, as well as collagen synthesis at respective doses of 1 and 10 μmol/L. Diclofenac sodium suppressed collagen synthesis of keratocytes in haze cornea at a high dose of 100 μmol/L, and in keratocytes in normal cornea, at doses of 10 and 100 μmol/L. In an in vivo study, either 0.5% tranilast, 0.1% betamethasone phosphate eye drops, or a tranilast base solution (control) was instilled four times daily to rabbits that had undergone PRK. Weekly evaluation of the inhibitory effect of these drugs on the development of haze was performed 2 weeks after surgery. Tranilast suppressed haze 6–13 weeks after PRK, but betamethasone phosphate showed no effect.

Conclusion: These results indicate that tranilast is potentially effective for inhibiting the corneal haze that occurs after PRK. **Jpn J Ophthalmol 1999;43:355–362** © 1999 Japanese Ophthalmological Society

Key Words: Corneal haze, in vitro, in vivo, photorefractive keratectomy, tranilast.

Introduction

Corneal haze (haze) is a potential postsurgical complication in excimer laser photorefractive keratectomy (PRK). Haze may cause a decrease in contrast sensitivity, development of halo and glare, predictability of the correction of refraction, and regression of the acquired correction.[1,2] It has been reported that permanent haze may occur in severe cases.[3] The therapy currently used for haze is steroid administration. However, Bergman and Spigelman,[4] and others[5–7] have reported adverse effects such as elevated intraocular pressure. The efficacy of steroids is still questionable. Nassaralla et al[8] have reported that topical diclofenac sodium was more effective than steroids for the prevention of haze. Histopathologically, corneal haze is characterized by the confluence and activation of keratocytes (fibroblasts) and the accumulation of abnormal collagen and other substances in the extracellular matrix.[4,9,10–14] However, at the present time, there are no definite conclusions concerning the cause and treatment of corneal haze.

Tranilast (chemical structure shown in Figure 1) is an anti-allergy drug (molecular weight = 327.34) that has an inhibitory effect on the release of chemi-

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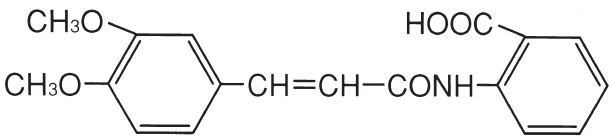

N-(3,4-dimethoxycinnamoyl) anthranilic acid

M.W. 327.34

Figure 1. Chemical structure of tranilast.

cal transmitters from mast cells, as well as a suppressive effect on vascular permeability.[15,16] The drug is used clinically, not only for allergic disease, but also to suppress collagen synthesis and the proliferation of keloid (scar formation by fibroblasts) and hypertrophic scar-derived fibroblasts.[16]

Based on the hypothesis that the inhibition of keratocyte activation and collagen synthesis in excimer laser–ablated cornea may prevent the development of corneal haze, excimer laser treatment (PRK) was performed on the eyes of pigmented rabbits to assess the in vitro effects of tranilast on the proliferation and collagen synthesis of cultured keratocytes from both haze and normal cornea. The in vivo therapeutic effect of tranilast eye drops on haze was also investigated.

Materials and Methods

Materials and Test Drugs

Male Dutch rabbits weighing about 2 kg each were used in this study. All experimental procedures conformed to the ARVO Resolution on the Use of Animals in Research. We used tranilast (Kissei Pharmaceutical, Matsumoto, Nagano), betamethasone phosphate (Sigma, St. Louis, MO, USA), and diclofenac sodium (Research Biochemicals International, Natick, MA, USA) as test drugs. These drugs were dissolved with dimethylsulfoxide (Nacalai Tesque, Kyoto) and appropriately diluted with Dulbecco's modified Eagle medium (DMEM; Gibco Laboratories, Grand Island, NY, USA). In addition, fetal bovine serum (FBS; Gibco Laboratories), porcine trypsin (trypsin; Wako Pure Chemical, Osaka), ethylenediamine–tetraacetic acid, disodium salt (EDTA; Wako Pure Chemical), β-aminopropionitrile (β-APN; Sigma), N-ethylmaleimide (Nacalai Tesque), collagenase form III (Advance Biofactures, Lynbrook, NY, USA), [2,3-^{3}H]proline ([^{3}H]proline; Dupont-NEN, Boston, MA, USA), and trypan blue (Wako Pure Chemical) were used in this study.

In Vitro Study

We performed bilateral PRK on 10 Dutch rabbits with excimer laser (Mini Excimer Compak-200®; Laser Sight Technology, Orlando, FL, USA). All animals were anesthetized with an intramuscular injection of ketamine (40 mg/kg) and xylazine (7 mg/kg). Before treatment, the corneal epithelium was removed and each cornea was dried completely with cellulose sponges.

PRK was carried out with a fluence of 0.85 mJ/pulse, and a pulse rate of 100 Hz in a 5.5-mm diameter area with a central depth of 64.89 mm, which corresponded to a correction of 10 diopters for myopia. After surgery ofloxacin ophthalmic ointment was applied to all eyes. Twenty-one days after PRK, we confirmed the development of corneal haze, and the rabbits were sacrificed using a lethal intravenous injection of pentobarbital. The corneal epithelium was removed, and then the haze regions in the stroma were harvested. In addition, five normal rabbits that did not undergo PRK were sacrificed and specimens of normal stroma were harvested in the same manner as described above.

Cultivation of keratocytes. Five-millimeter-square fragments of normal and haze corneal specimens were prepared, treated in DMEM containing 0.4% collagenase, and then cultured in a 75-mL flask. The culture medium consisted of DMEM with 20% FBS, penicillin (50 IU/mL), streptomycin (50 μg/mL), and kanamycin (50 μg/mL). Culturing was performed at 37°C in 5% CO_2. The culture medium was changed every 3 or 4 days. When the bottom of the flask was covered with cells, the cells were peeled off using 0.25% trypsin–0.02% EDTA and subcultured. The subculture medium was DMEM containing 10% FBS. The 5th generation subculture was used.

Proliferation of cultured keratocytes. Six-well plates were inoculated with 10^5 keratocytes from normal or haze corneas and incubated for 24 hours at 37°C. The cells were divided into three groups and cultured for 24, 48, or 72 hours. Next, the cells were inoculated in the same manner as described above, the medium was changed to 10% FBS-DMEM containing one of the test drugs, and the cells were cultured for a further 48 hours. The cultured cells were treated with 0.25% trypsin–0.02% EDTA, and the number of cells was determined using a hemocytometer.

Collagen synthesis. Six-well plates were inoculated with 10^5 keratocytes from normal or haze corneas and

incubated for 6 hours at 37°C in DMEM containing β-APN (100 mg/mL), [^{3}H]proline (37 kBq/mL), and one of the test drugs. The amount of collagen in the supernatant and cell homogenate was determined using Peterkofsky and Diegelmann's cellular collagenase digestion technique[17] as follows. Equal volumes of 10% trichloroacetic acid (TCA)–0.5% tannic acid and FBS were added to the cell supernatant and cell homogenate, and the mixtures were allowed to stand for 10 minutes before centrifuging at 4°C (3,000 *g* for 5 minutes). The precipitates were washed twice with 5% TCA–0.25% tannic acid, cooled by cold acetone, and then dried. The dried precipitates were dissolved in 0.1 N NaOH, neutralized, and then reacted with collagenase (14 BTC U/mL) in the presence of 25 mmol/L of N-ethylmaleimide and 50 mmol/L of $CaCl_2$ at 37°C for 3 hours. Equal volumes of 10% TCA–0.5% tannic acid and FBS were added to the mixture, which was then centrifuged (3,000 *g* for 5 minutes). Finally, the radioactivity of the collagen-type protein in the supernatant was measured using a spectrofluorometer and considered as the amount of DNA in the cell homogenate.

In Vivo Study

We performed bilateral PRK on 21 Dutch rabbits using the same procedures as in the in vitro study. The rabbits were divided into three groups depending on the postoperative regimen. The tranilast group (T group) was given 0.5% tranilast eye drops; betamethasone phosphate group (BP group) was given 0.1% betamethasone phosphate eye drops; and control group (C group) was given tranilast base solution only. The composition of a 0.5% tranilast eye drop is shown in Table 1. By micropipette, the rabbits in each group received bilateral instillations of their eye drops (50 mL) four times per day (9 A.M., 12 P.M., 3 P.M., and 6 P.M.), starting the day after surgery for 13 consecutive weeks.

Using a slit-lamp microscope, the inhibitory effect of these drugs on haze was evaluated according to the Fantes' classification[11] every week from 2 to 13 weeks after surgery. Grade 0 corresponds to a totally clear cornea; grade 0.5 corresponds to a trace of corneal opacification visible by broad tangential illumination; grade 1 corresponds to a minimal-density haze seen with direct or tangential illumination; grade 2 corresponds to a mild haze easily seen by direct illumination; grade 3 corresponds to a moderate haze that partially obscures iris details; and grade 4 corresponds to a severe haze that completely obscures iris details. All clinical evaluations were carried out by the same masked observer.

Statistical Analysis

In the in vitro study, each value indicated the mean ± standard error of the mean (SEM). The unpaired Student's *t* test was used to determine the cellular proliferative capability. In the other experiments, analysis of variance and then Fisher's least significant difference test were used. In the in vivo study, each haze score was expressed as mean ± SEM. The Kruskal-Wallis test and Fisher's least significant difference test were used. The P values $<.05$ were considered statistically significant.

Results

In Vitro Study

The proliferative capability of keratocytes isolated from the corneal haze region was signficantly greater than that of keratocytes from normal cornea at 24, 48, and 72 hours after culturing (Figure 2).

Tranilast suppressed the proliferation of keratocytes from haze cornea at doses of 30 and 300 μmol/L dose dependently (Figure 3A), and the proliferation of keratocytes from normal cornea only at the high dose of 300 μmol/L (Figure 3B).

Figure 4 illustrates that there was less cell proliferation of keratocytes from haze cornea in the tranilast group compared with the control group.

Betamethasone suppressed proliferation of keratocytes from both haze and normal cornea at a dose of 10 μmol/L (Figure 3).

Table 1. Composition of Tranilast Eye Drop

Tranilast 0.5%	
Contains	Boride as buffer and benzalkonium chloride as preservative
Character	Slightly yellow and clear
pH	7.0–8.0
Ratio of penetration	About 1 (compared with 0.9% NaCl)

Jpn J Ophthalmol
Vol 43: 355–362, 1999

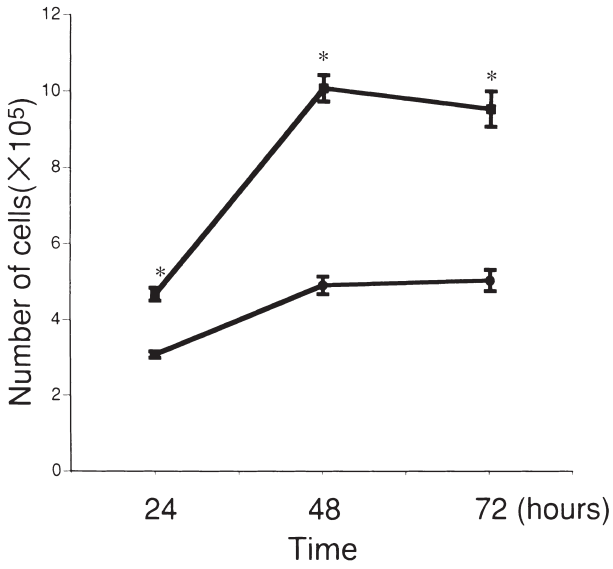

Figure 2. Cellular proliferative capability of cultured rabbit keratocytes. Each value indicates mean ± SEM (n = 6). *Significant difference from control at $P < .001$. ■: keratocytes in haze cornea; ●: keratocytes in normal cornea.

Diclofenac sodium at either dose did not suppress the proliferation of keratocytes from either haze or normal cornea (Figure 3).

Collagen synthesis. Tranilast significantly suppressed the collagen synthesis of keratocytes in haze cornea at doses of 3 to 300 μmol/L (Figure 5A), but affected collagen synthesis of keratocytes in normal cornea only at the high dose of 300 μmol/L (Figure 5B).

Betamethasone suppressed proliferation and collagen synthesis of keratocytes in both haze and normal cornea at respective doses of 1 and 10 μmol/L (Figure 5).

Diclofenac sodium suppressed the collagen synthesis of keratocytes in haze cornea only at the high dose of 100 μmol/L (Figure 5A), but of keratocytes in normal cornea at doses of 10 and 100 μmol/L (Figure 5B).

In Vivo Study

The ablated corneal area was reepithelialized completely in all rabbits by 10 days after surgery. However, reepithelialization was slightly slower in the BP group compared with the T group. The changes in the Haze score are shown in Figure 6. In all three groups, the Haze score tended to increase 3 weeks after surgery. A peak was seen at 5–7 weeks, followed by a decrease. For each time period, the degree of haze was less for the T group than for the

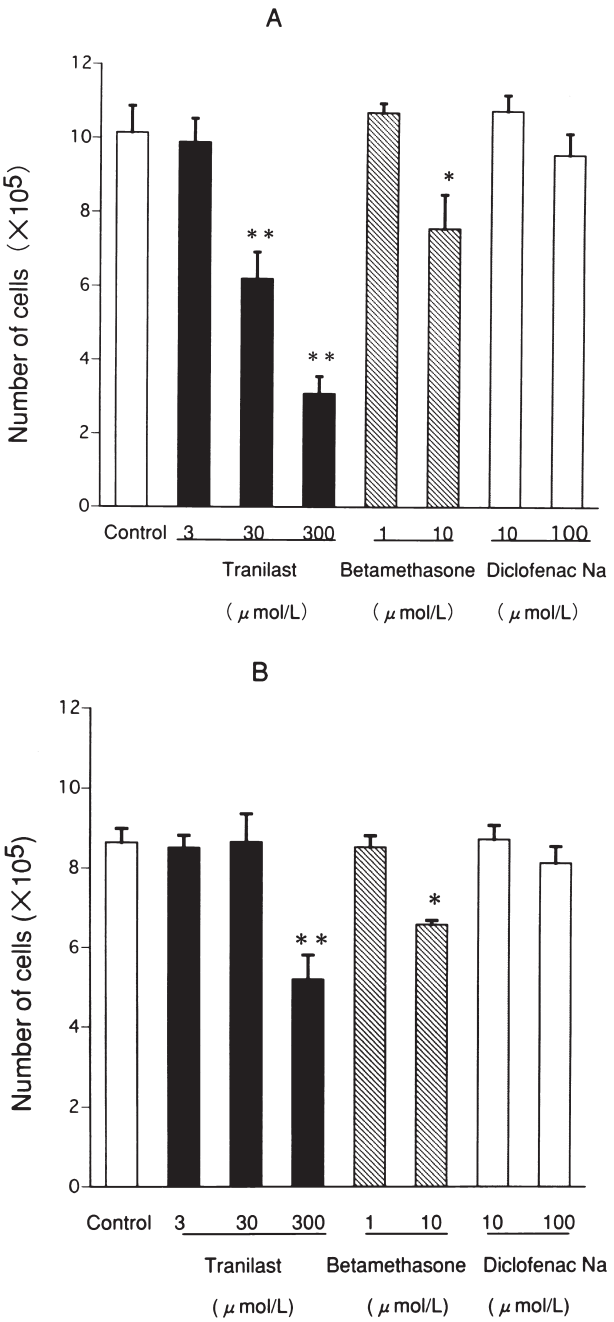

Figure 3. Effect of each drug on cellular proliferative ability of cultured rabbit keratocytes. Each column indicates mean ± SEM (n = 4).* and **: significant difference from control at $P < .01$ and $P < .001$, respectively. (**A**) Keratocytes in corneal haze region. (**B**) Keratocytes in normal cornea.

other two groups. The difference was significant at 3 and 6 weeks, and each week subsequent to week 6 ($P < .01$ for weeks 8, 11, 12, and 13; and $P < .05$ for weeks 3, 6, 7, 9, and 10). Haze was not reduced in the

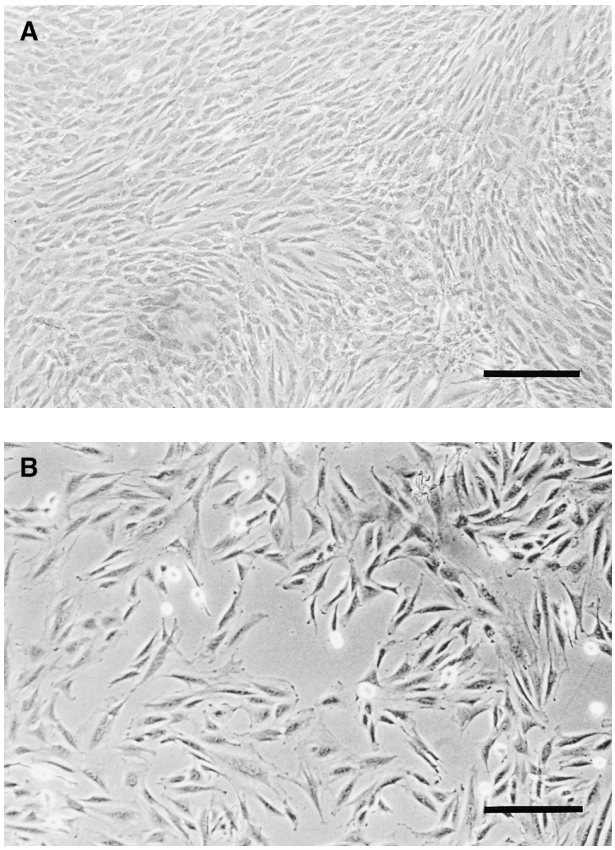

Figure 4. Phase-difference photomicrograph (cultured keratocytes from corneal haze region) Proliferation of keratocytes in haze specimen was less in tranilast group (**B**) (300 μmol/L) than in control (**A**). Bar: 50 μm.

BP group. Typical examples of haze in each of the three groups at 6 and 13 weeks after surgery are shown in Figure 7.

Discussion

Corneal haze is a potential complication in PRK. A previous study showed that the epithelium was regenerated 4–6 days after the stromal ablation by excimer laser.[13] The ablated corneal stroma was reconstructed by activation of the surrounding keratocytes (converted to fibroblasts), that comprised the confluence of keratocytes and the production of extracellular matrixes such as collagen type III and proteoglycan.[4,10–14] The synthesized collagen fiber showed irregular distribution[13] that reduced the transparency of the cornea. This is clinically referred to as haze. This process might be controlled by growth factors and cytokines, as in other tissue such as skin keloids and hypertrophic scars.[18] Although topical steroid treatment is now generally used to treat haze,

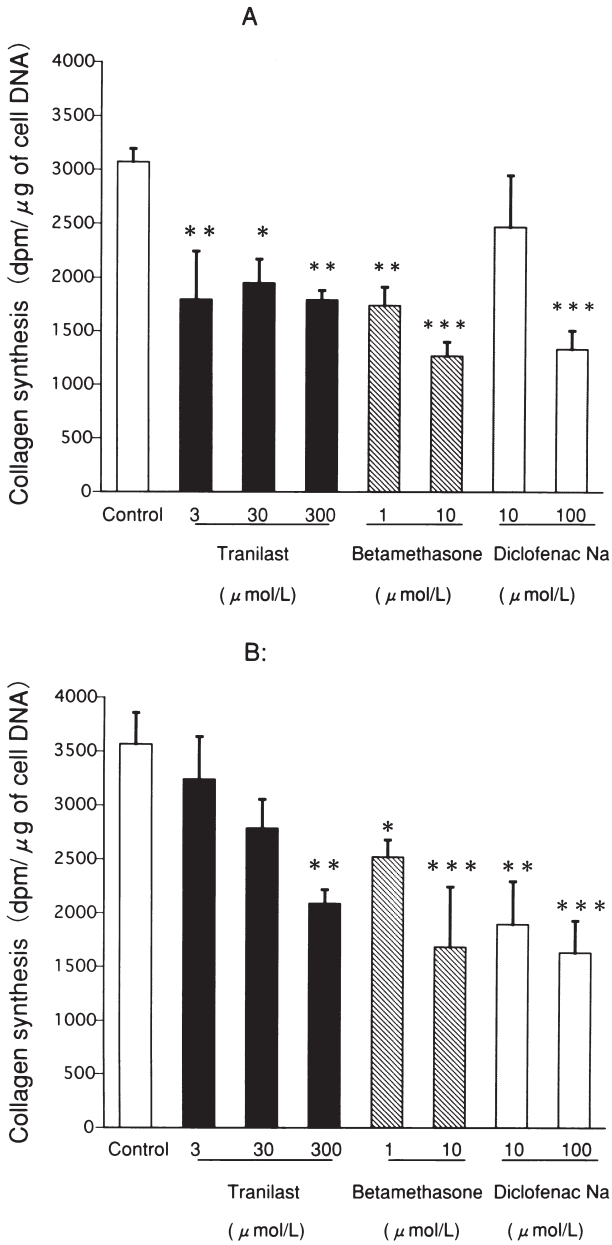

Figure 5. Effect of each drug on collagen synthesis in cultured rabbit keratocytes. Each column indicates mean ± SEM (n = 4). *, **, ***: significant difference from control at $P < .05$, $P < .01$, and $P < .001$, respectively. (**A**) Keratocytes in corneal haze region. (**B**) Keratocytes in normal cornea.

it has adverse effects, such as elevation of intraocular pressure or lowering of resistance to infection, and is thought to be not so effective. Therefore, no definite conclusion has been reached for the etiology and treatment of haze.

Tranilast, as an oral anti-allergy drug, suppresses vascular permeability and releases chemical trans-

Jpn J Ophthalmol
Vol 43: 355–362, 1999

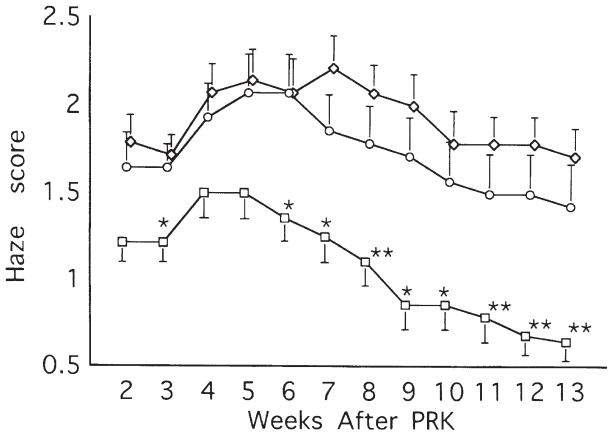

Figure 6. Effect of tranilast on corneal haze ○: 0.5% tranilast base solution group; □: 0.5% tranilast eye drop group; ◇: 0.1% betamethasone phosphate eye drop group. Each value indicates mean ± SEM. See text for explanation of evaluation. Significant difference from control $*P < .05$, $**P < .01$.

mitters from inflammatory cells such as mast cells.[19] On the other hand, tranilast is effective not only as an anti-allergy drug, but also for suppression of proliferation and collagen synthesis by fibroblasts (keloid) and hypertrophic scar-derived fibroblasts.[16] The tranilast mechanism is believed to inhibit the production and release of cytokines such as TGF-β1 from fibroblasts as well as to suppress a release of chemical transmitters from inflammatory cells.[15] These pharmacological effects can be applied to treating diseases in which fibroblasts play a role. Tranilast has been used clinically to treat or prevent scar formation.

The present study showed that tranilast suppressed the proliferation of cultured keratocytes from haze cornea in vitro. It also prevented haze in vivo. Because inflammatory cells, such as mast cells, were not involved in this study, tranilast may have inhibited the production and release of cytokines like TGF-β1 from keratocytes. The in vitro study comparing keratocytes in haze and normal corneas showed that cellular proliferation was significantly greater in keratocytes in haze cornea. Tranilast suppressed the proliferation and collagen synthesis of keratocytes in haze cornea at lower doses than needed for keratocytes in normal cornea (30 and 3 μmol/L vs. 300 μmol/L). The dose of 300 μmol/L is approximately three times higher than the maximum therapeutic dose of the 0.5% tranilast eye drops (about 98 μmol/L).[20] A recent study has reported that fibroblasts produced a greater amount of TGF-β1 in keloids and hypertrophic scars than in normal skin tissue.[21] Therefore, the keratocytes in haze cornea may produce a greater amount of cytokines including TGF-β1, just as fibroblasts do in keloids and hypertrophic scars. This may explain the greater cell proliferation and increased effectiveness of tranilast at lower doses for keratocytes in haze cornea. Unlike betamethasone, tranilast at clinical doses specifically suppressed keratocyte proliferation and collagen synthesis of keratocytes in haze cornea but did not affect keratocytes in normal cornea.

Steroids generally inhibit DNA synthesis and collagen biosynthesis of fibroblasts by nonspecific antimetabolic effects.[14] You et al,[22] reported that steroid eye drops prevented haze by inhibiting the confluence of fibroblasts to the epithelial–stromal junction. However, our study showed that betamethasone phosphate did not prevent haze in vivo, although it suppressed the proliferation and collagen synthesis of keratocytes in haze and normal cornea in vitro at the same concentrations of 10 μmol/L and 1 μmol/L, respectively. The maximum corneal concentration with 0.1% betamethasone phosphate eye drops was about 3.4 μmol/L,[23] which should be effective in suppressing collagen synthesis, but not in suppressing keratocyte proliferation.

Gartry et al[2] and Obart et al[7] have reported that steroids did not inhibit haze in rabbits, possibly because the type of collagen in rabbit and human cornea is different. Other studies point out that the interaction between the regenerated epithelium and stroma in wound healing may be involved in the etiology of corneal haze.[11,12] Delayed healing of the corneal epithelium may also be involved.

Diclofenac sodium is considered to inhibit the biosynthesis of prostaglandin by inhibiting cyclooxygenase.[8] Nassaralla et al[8] reported that diclofenac sodium eye drops were effective in suppressing or preventing haze in rabbits. In our study, however, they suppressed collagen synthesis of keratocytes in haze and normal cornea at 100 μmol/L, but did not suppress their proliferation. Because the maximum corneal concentration of the clinically used 0.1% diclofenac sodium eye drops was about 43 μmol/L,[24] diclofenac sodium may not have had an inhibitory effect on the proliferation and collagen synthesis of keratocytes in haze cornea. Based on these results, tranilast is considered effective in preventing haze after excimer laser surgery.

A part of this study (the in vivo study) was published in Japanese in Nippon Ganka Gakkai Zasshi (J Jpn Ophthalmol Soc) 1997;

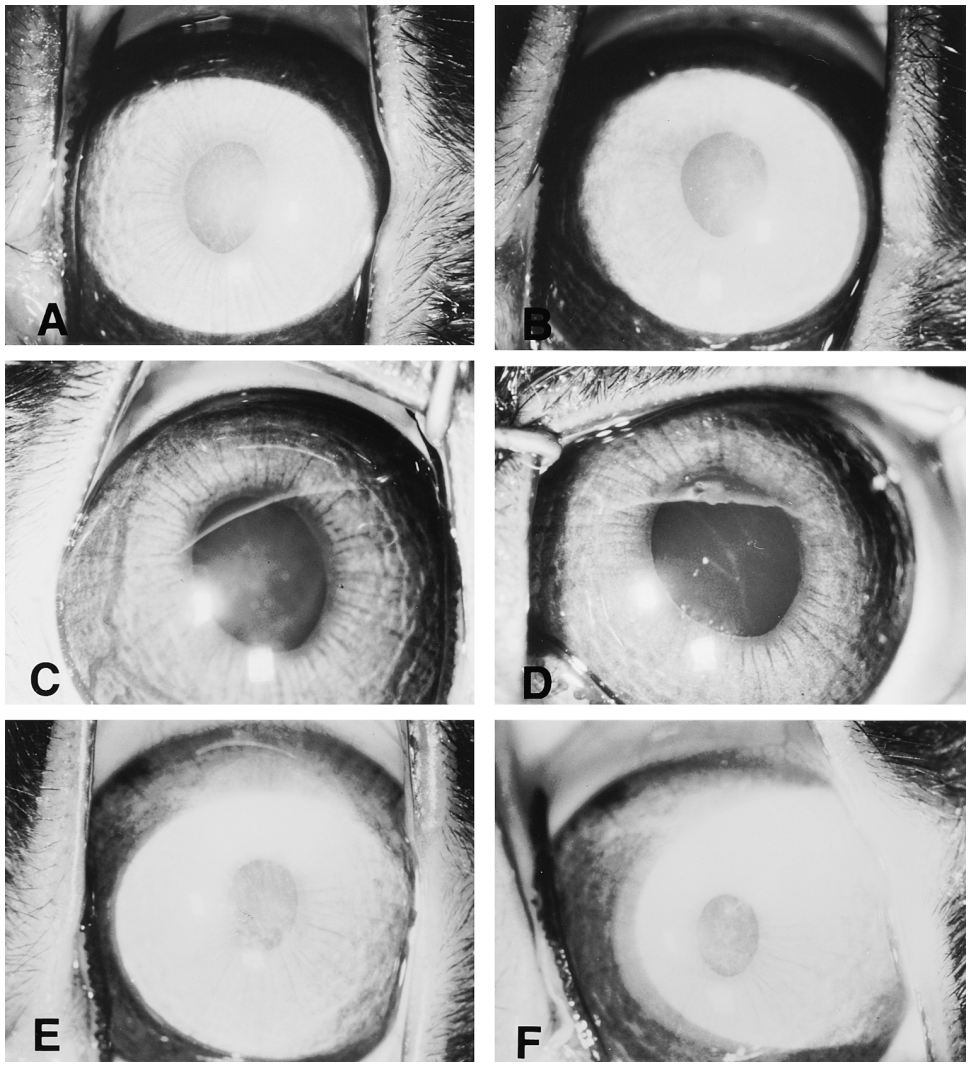

Figure 7. (**A** and **B**) 0.5% tranilast base solution group (control). (**C** and **D**) tranilast eye drop group. (**E** and **F**) 0.1% betamethasone phosphate eye drop group. Groups A, C, and E: 6 weeks after PRK; B, D, and F: 13 weeks after PRK. 0.5% tranilast suppressed corneal haze.

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