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Effects of Various Eye Drops on Corneal Wound Healing after Superficial Keratectomy in Rabbits

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Purpose: The effects of various eye drops on corneal wound healing, particularly in the subepithelial haze area, were investigated histologically following superficial keratectomy in rabbits.

Methods: Mechanical superficial keratectomy was performed in rabbit eyes. Tranilast, betamethasone, hyaluronic acid, and diclofenac eye drops were administered after the procedure. Physiological saline was used as a control. Corneas were excised 1, 2, 3, and 4 weeks after keratectomy, labeled with ^{3}H-thymidine or ^{3}H-proline, and subjected to autoradiography.

Results: In the control and diclofenac groups, corneal haze occurred 3 weeks after keratectomy. Histological examination revealed an accumulation of proliferating keratocytes and active synthesis of collagen in the subepithelial area. In the tranilast and betamethasone groups, formation of corneal haze was reduced compared to the controls. The proliferation of keratocytes and the production of collagen in the corneal stroma were inhibited by these drugs. In the hyaluronic acid group also, corneal haze was decreased. In this group, although the proliferation of keratocytes was activated compared to the controls, abnormal accumulation of keratocytes in the subepithelial area was not detected.

Conclusions: Tranilast and betamethasone decrease the formation of subepithelial haze by inhibiting keratocyte proliferation and synthesis of extracellular matrix in the corneal stroma. Hyaluronic acid, on the other hand, inhibits subepithelial haze by promoting physiologic wound healing. **Jpn J Ophthalmol 2002;46:488–495** © 2002 Japanese Opthamological Society

Key Words: Corneal wound healing, hyaluronic acid, subepithelial haze, superficial keratectomy, tranilast.

Introduction

Subepithelial haze is one of the major complications of excimer laser superficial keratectomy. It has been reported that subepithelial opacity occurs in most patients after phototherapeutic keratectomy (PTK) or photorefractive keratectomy (PRK), resulting in permanent visual disturbance in the more severe cases.[1] Although the prevention of subepithelial haze is a critical issue with PTK or PRK, little is known about the underlying mechanisms of its development. Subepithelial haze after PTK or PRK is reported to be qualitatively identical to the corneal haze that develops after mechanical superficial keratectomy.[2,3] Corneal transparency is maintained by the regularity in spacing and uniformity in diameter of the collagen fibrils in the corneal stroma, with fibril spacing being less than half the wavelength of light. Proteins such as glycoproteins and proteoglycans occupy the spaces between the collagen fibrils. With corneal injury, proliferation of keratocytes in the stroma with excess production of extracellular matrix, including collagen fibrils, occurs.[4–6] The abnormal accumulation of extracellular matrix also re-

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sults in the development of corneal stromal haze after superficial keratectomy.[7,8]

Steroids, nonsteroidal anti-inflammatory drugs, and hyaluronic acid have all been clinically used to prevent inflammation, to ease pain, or to promote epithelial regeneration after ocular surgery. Recently, tranilast, an anti-allergy agent, has also been shown to inhibit subepithelial haze after PRK.[9,10] The effects of these drugs on corneal wound healing, however, have not yet been fully clarified. In this study, we investigated the effects these drugs would have on corneal wound healing in rabbits after mechanical superficial keratectomy.

Materials and Methods

Animals and Experimental Procedures

Twenty female adult albino rabbits (Japanese White) each weighing 2.5 kg were used in this study. All rabbits were treated under the Guidelines for Animal Research at Kobe University Graduate School of Medicine, and the ARVO resolution on the Care and Use of Animals in Vision Research. Rabbits were initially anesthetized with intramuscular ketamine (30 mg/kg) before the application of 0.4% oxybuprocaine eye drops. A superficial keratectomy (6 mm diameter) was done in the central area of the cornea using a sterile, single-use, no.15 disposable scalpel (Feather Safety Razor, Osaka). Rabbits were divided into five groups according to the drug administered; Control group (physiological saline), Tranilast group, Betamethasone group, Hyaluronic acid group, and Diclofenac group. After keratectomy, the ophthalmic solutions were administered topically three times a day until shortly before sacrifice. Topical antibiotics were not used. After macroscopic observation of the corneal haze formation and epithelial defects, rabbits were sacrificed with an overdose of intravenous sodium pentobarbital at 1, 2, 3, and 4 weeks post-keratectomy. Corneas were excised, cut in half at the middle and labeled with ^{3}H-thymidine (10 μCi/mL, Amersham Japan, Tokyo) or ^{3}H-proline (10 μCi/mL, Amersham Japan) at 37°C for 4 hours, and processed for autoradiography. The corneas were fixed and paraffin sections (3 μm thick) were mounted on glass slides. The slides were dipped in Konica NR-M2 emulsion (Konica, Tokyo), dried, and exposed in the dark at 4°C for 3–5 weeks. The slides were then developed with Fuji Lendole (Fuji, Tokyo), fixed with Fuji Lenfix, stained with hematoxylin and eosin and examined under a light microscope.[11] Several slides of the Control group were stained with toluidine blue for histological studies of subepithelial scar tissue formation.

Data Analysis

For quantitative analysis of the relative effect of each drug on the proliferation of keratocytes, we calculated the average number of ^{3}H-thymidine-incorporated keratocytes for five specimens per group, and then standardized it by the length of each specimen. Results were expressed as mean ± SEM. Comparisons between each group and the control were made using Dunnett's multiple comparison procedure.[12] All findings were considered significant when the P value was $< .05$.

In a subset of experiments on ^{3}H-proline autoradiography, we compared the density of silver grains in ^{3}H-proline-incorporated keratocytes imaged on the photomicrographs to compare the relative effects of drugs on collagen synthesis in keratocytes.

Drugs

The following drugs were used in this study: 0.5% tranilast (Rizaben®, Kissei, Nagano), 0.1% betamethasone sodium phosphate (Rinderon®, Shionogi, Osaka), 0.1% hyaluronic acid (Hyalein®, Santen, Osaka), 0.1% diclofenac sodium (Dichlod®, Wakamoto Tokyo), ketamine hydrochloride (Ketalar®,

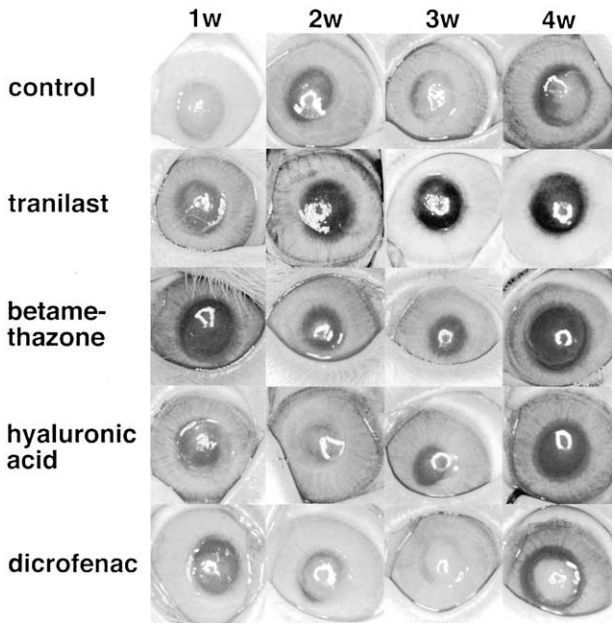

Figure 1. Macroscopic findings in the anterior segments. Corneal epithelium regenerated at 2 weeks post-keratectomy in all groups. In Control and Diclofenac groups, corneal haze at the site of the wound occurred 3 weeks post-keratectomy, and became severe at 4 weeks. In Tranilast and Hyaluronic acid groups, corneal haze was dramatically inhibited, compared to Control group. Betamethasone group showed little or no haze 4 weeks post-keratectomy.

Jpn J Ophthalmol
Vol 46: 488–495, 2002

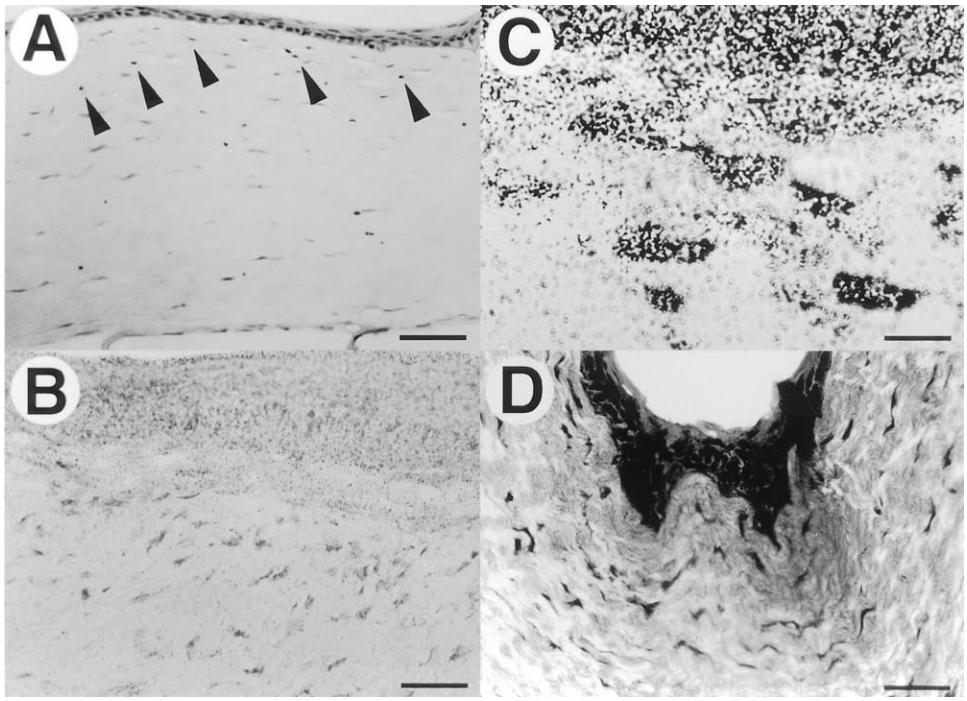

Figure 2. Microscopic findings in the control corneas. (**A**) ^{3}H-thymidine autoradiogram, 2 weeks post-keratectomy. Epithelial cells at the injured area regenerated forming two to three cell layers. In the stroma, infiltration of keratocytes in the subepithelial acellular area progressed. Numerous ^{3}H-thymidine-incorporating keratocytes (arrowheads) were observed in the stroma. Bar = 60 μm. (**B**,**C**) ^{3}H-proline autoradiograms 3 weeks post-keratectomy. Keratocytes accumulated in the subepithelial area. A high-density ^{3}H-proline keratocyte accumulation was observed in the stroma, indicating active collagen synthesis. Bar = 60 μm in B, and 20 μm in C. (**D**) The area of cornea within the haze was positive for toluidine blue, indicating the presence of scar tissue. Bar = 60 μm.

Sankyo, Tokyo), and 0.4% oxybuprocaine hydrochloride solution (Benoxil®, Santen).

Results

Macroscopic Findings of the Anterior Segment

Macroscopic observation at 1 week post-keratectomy demonstrated complete corneal epithelial regeneration in the Control, Tranilast, and Hyaluronic acid groups, with only partial regeneration in the Betamethasone and Diclofenac groups. At 2 weeks post-keratectomy, complete epithelial regeneration was observed in all groups.

In the Control and Diclofenac groups, corneal haze at the site of the wound developed at 3 weeks post-keratectomy, becoming more severe after 4 weeks. In the Tranilast and Hyaluronic acid groups, corneal haze formation was inhibited. The Betamethasone group showed little or no haze at 4 weeks post-keratectomy (Figure 1).

Microscopic Findings

Control group. Figure 2A shows the ^{3}H-thymidine autoradiogram 2 weeks after keratectomy. Epithelial cells at the injured area had regenerated forming two to three layers. In the stromal layer, keratocyte infiltration into the subepithelial acellular area was observed. Numerous ^{3}H-thymidine-incorporated keratocytes were seen in the stroma, indicating active cell proliferation (Figure 2A, arrowheads). The number of ^{3}H-thymidine-incorporated keratocytes was 92.0 ± 3.4 (per 1 cm-long corneal tissue). Figures 2B and 2C show the 3-week post-keratectomy ^{3}H-proline autoradiograms. Keratocytes accumulated in the subepithelial area. High-density ^{3}H-proline-incorporating keratocytes were observed in the stroma indicating active collagen synthesis. The area of keratocyte accumulation stained positive with toluidine blue, indicating that scar tissue formation accounted for the subepithelial haze (Figure 2D).

Tranilast group. Epithelial cells regenerated from 2 weeks post-keratectomy, forming two to three layers of cells over the injured area. Infiltration of keratocytes into the acellular area in the subepithelial portion was delayed (Figure 3A). Keratocyte uptake of ^{3}H-thymidine was significantly decreased compared with the controls (26.2 ± 1.3 vs. 92.0 ± 3.4, $P < .01$) (Figure 7). Keratocyte infiltration persisted to the

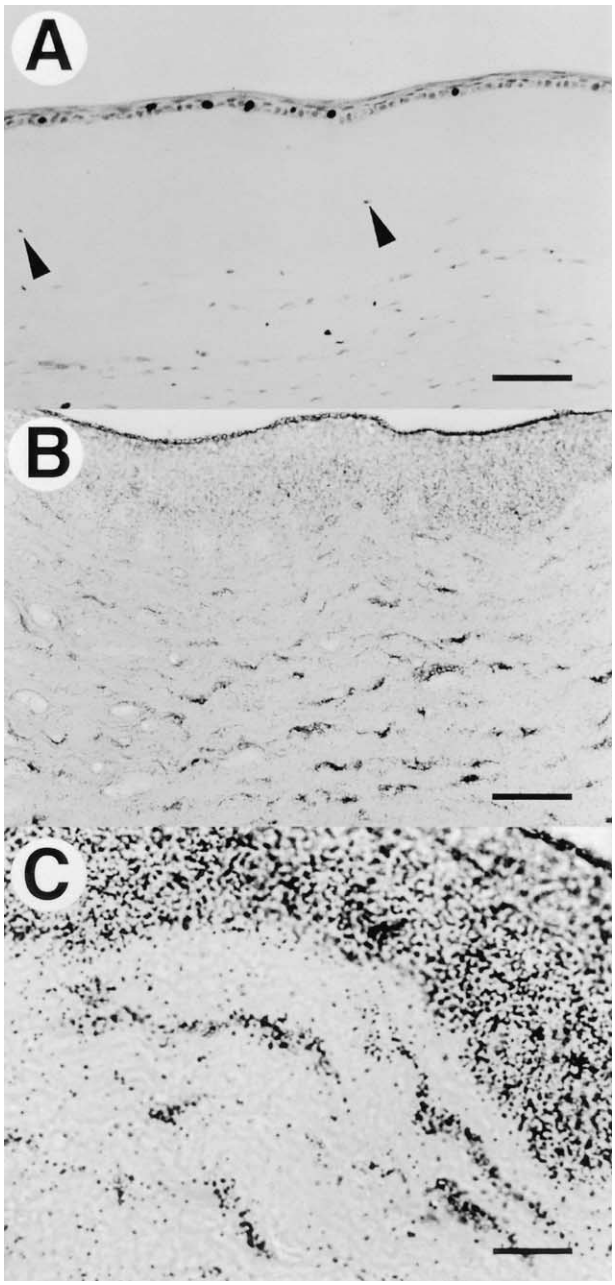

Figure 3. Microscopic findings in the tranilast-treated corneas. (**A**) ^{3}H-thymidine autoradiograms, 2 weeks post-keratectomy. Two to three layers of epithelial cells regenerated over the wound. Infiltration of keratocytes in the acellular subepithelial area was delayed and the uptake of ^{3}H-thymidine by keratocytes was decreased (arrowheads). Bar = 60 μm. (**B**,**C**) ^{3}H-proline autoradiograms, 3 weeks post-keratectomy. Infiltration of keratocytes progressed. There was no accumulation of keratocytes in the subepithelial area. Keratocyte uptake of ^{3}H-proline was attenuated compared to Control group. Bar = 60 μm in (**B**), and 20 μm in (**C**).

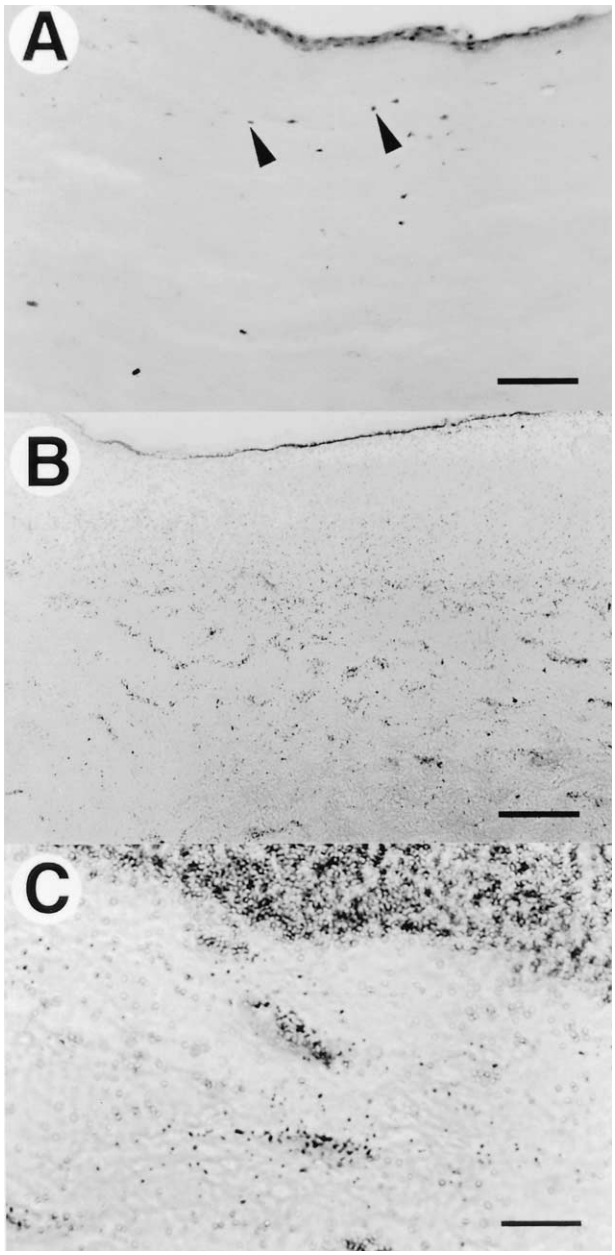

Figure 4. Microscopic findings in the betamethasone-treated corneas. (**A**) ^{3}H-thymidine autoradiograms, 2 weeks post-keratectomy. The epithelial layers regenerated, but were thinner than those in Control group. Infiltration of keratocytes toward the acellular area in the subepithelial portion was delayed, and few keratocytes incorporated ^{3}H-thymidine (arrowheads). Bar = 60 μm. (**B**,**C**) ^{3}H-proline autoradiograms, 3 weeks post-keratectomy. Infiltration of keratocytes progressed but accumulation of keratocytes was inhibited. Uptake of ^{3}H-proline by keratocytes was inhibited. Bar = 60 μm in (**B**), and 20 μm in (**C**).

Jpn J Ophthalmol
Vol 46: 488–495, 2002

3-week post-keratectomy period (Figures 3B and 3C). The accumulation of keratocytes in the subepithelial area was not as dense as in the Control group, and uptake of ^{3}H-proline by keratocytes was attenuated compared with the Control group (Figures 3B and 3C).

Betamethasone group. Epithelial cells regenerated 2 weeks post-keratectomy. However, the regenerated epithelial layers were relatively thinner than those in the Control group. Infiltration of keratocytes into the acellular area in the subepithelial portion was delayed (Figure 4A), and the number of keratocytes incorporating ^{3}H-thymidine was significantly decreased (45.4 ± 1.6 vs. 92.0 ± 3.4, $P < .01$) (Figure 7). Infiltration of keratocytes persisted at 3 weeks post-keratectomy. The accumulation of keratocytes was less than that in the Control group, and the uptake of ^{3}H-proline by keratocytes was inhibited (Figures 4B and 4C).

Hyaluronic acid group Epithelial cells regenerated within 2 weeks and formed 2 to 3 layers. Infiltration into the epithelial layer progressed. Numerous ^{3}H-thymidine incorporating keratocytes were observed in the stroma (Figure 5A). The number of ^{3}H-thymidine-incorporated keratocytes per 1 cm length of corneal tissue was 170.2 ± 4.8 compared with 92.0 ± 3.4 in the Control group ($P < .01$) (Figure 7). Although the density of keratocytes seemed high at 3 weeks post-keratectomy, the accumulation was less than that in the Control group (Figures 5B and 5C). Uptake of ^{3}H -proline was almost at the same level as that in the Control group.

Diclofenac group. Epithelial cells regenerated at 2 weeks post-keratectomy. The regenerated epithelium formed 1 to 2 layers, which appeared thinner than those in the other groups. The stromal layer was thick and edematous, with remarkable infiltration of inflammatory cells and neovascularization. Formation of a retrocorneal membrane was also observed on the posterior surface of the endothelium (Figure 6A). The number of ^{3}H-thymidine-incorporated keratocytes per 1 cm length of corneal tissue was significantly decreased (72.2 ± 4.1) compared with the Control group (92.0 ± 3.4, $P < .01$) (Figure 7). ^{3}H-proline uptake was similar to that of Control group (Figures 6B and C).

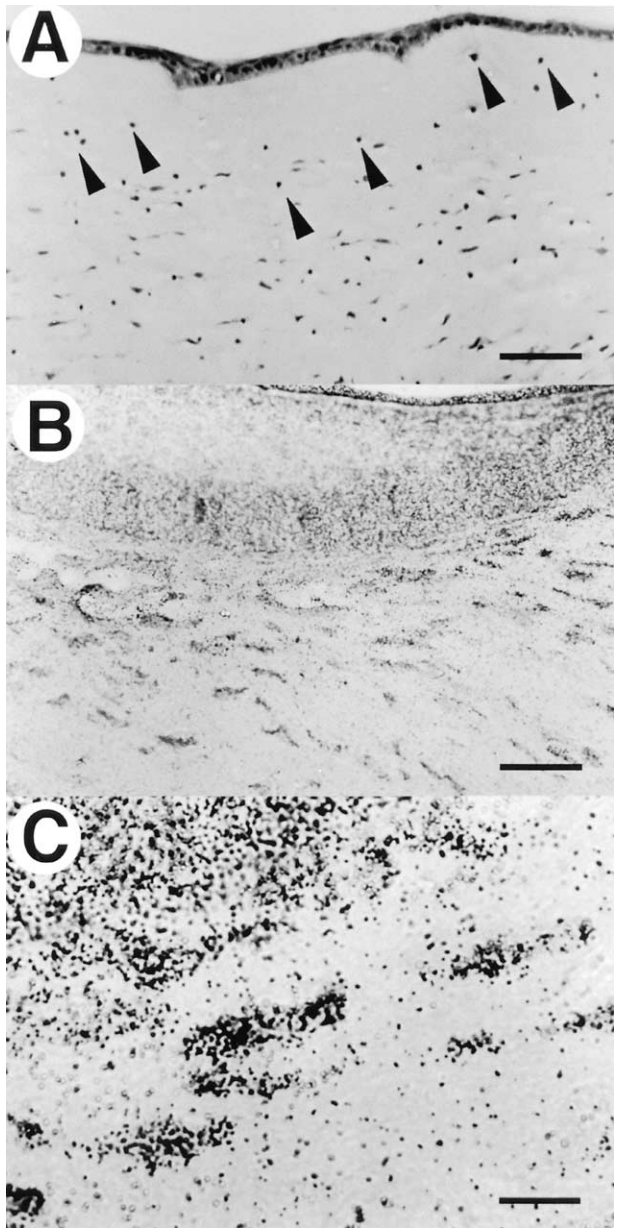

Figure 5. Microscopic findings in the hyaluronic acid-treated corneas. (**A**) ^{3}H-thymidine autoradiograms, 2 weeks post-keratectomy. Epithelial cells regenerated and formed two to three layers. Infiltration of keratocytes under the epithelial layer progressed. Numerous ^{3}H-thymidine incorporated keratocytes were observed (arrowheads) in the stroma. Bar = 60 μm. (**B**,**C**) ^{3}H-proline autoradiograms, 3 weeks post-keratectomy. Although the density of keratocytes seemed high, there was no abnormal accumulation, as seen in Control group. Keratocyte uptake of ^{3}H -proline was almost equal to that in Control group. Bar = 60 μm in (**B**), and 20 μm in (**C**).

Discussion

In the present study, we examined the histological effects of tranilast, betamethasone, diclofenac, and hyaluronic acid on rabbit corneal wound healing, especially at the area of subepithelial haze formation,

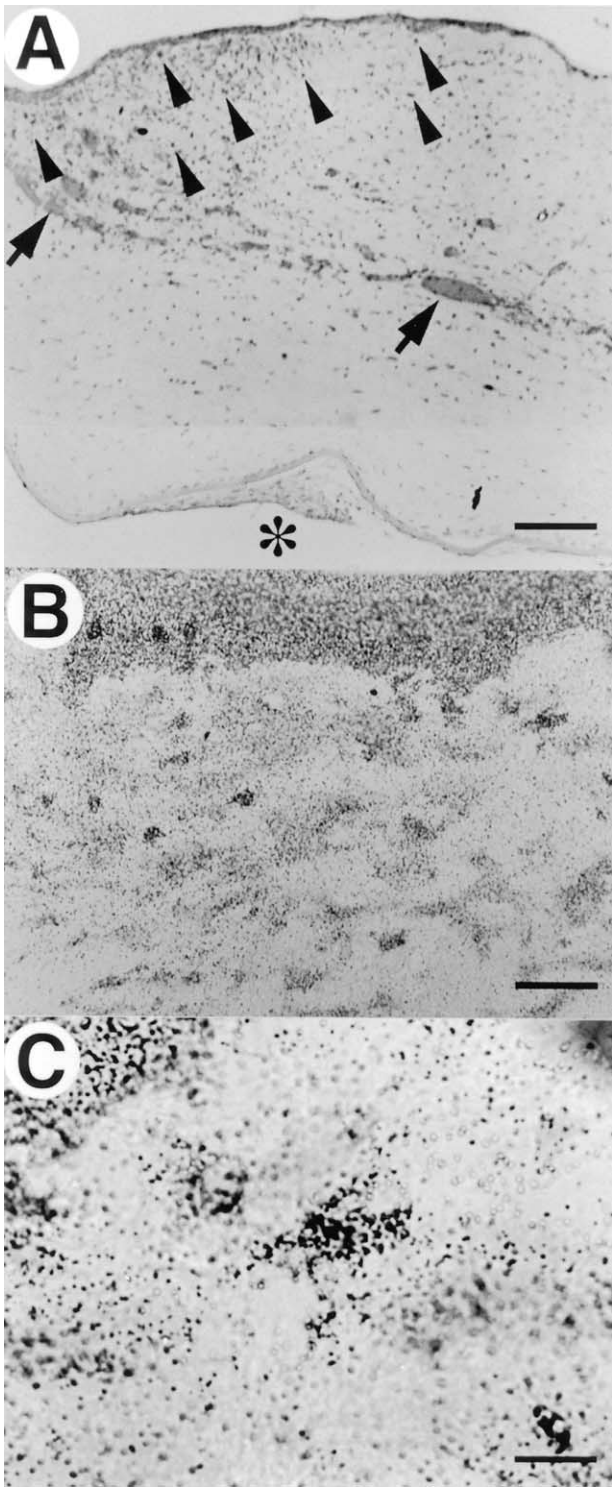

Figure 6. Microscopic findings in the diclofenac-treated corneas. (**A**) ^{3}H-thymidine autoradiograms, 2 weeks post-keratectomy. Epithelial cells regenerated forming one to two layers, which appeared thinner than those in other groups. The stromal layer became thick and edematous, with remarkable inflammatory cell infiltration and neovascular formation (arrows). The arrowheads demonstrate incorporation of ^{3}H-thymidine in keratocytes. Formation of a retrocorneal membrane was observed at the posterior surface of the endothelial cell layer (asterisk). Bar = 60 μm. (**B**,**C**) ^{3}H-proline autoradiograms 3 weeks post-keratectomy. Uptake of ^{3}H-proline by keratocytes was almostequal to that of Control group. Bar = 60 μm in (**B**), and 20 μm in (**C**).

following superficial keratectomy. Corneal haze formation was observed in the Control group at 3 weeks post-keratectomy. Histological examination revealed an accumulation of keratocytes and the active synthesis of collagen fibers at the subepithelial area. The corneal haze was markedly inhibited in the Betamethasone and Tranilast groups. The proliferation of keratocytes and synthesis of collagen fibers were significantly inhibited in these groups. Corneal haze was also inhibited in the Hyaluronic acid group. In contrast to the Betamethasone and Tranilast groups, the proliferation of keratocytes in the Hyaluronic acid group was somewhat augmented when compared with the Control group. These findings indicate that betamethasone, tranilast, and hyaluronic acid decreased subepithelial haze formation after superficial keratectomy, and that the inhibitory effects of these drugs are mediated by distinct mechanisms. Tranilast and betamethasone both inhibit keratocyte proliferation and an abnormal accumulation of extracellular matrix, while hyaluronic acid is likely to promote physiologic wound healing.

Tranilast is an anti-allergic agent that blocks histamine release from mast cells and basophils, and subsequently reduces vascular permeability. Previous studies indicated that this drug also inhibits fibroblast proliferation and collagen synthesis.[10] It has been postulated that several cytokines, which are produced at the site of the injured stroma, play crucial roles in the healing process after keratectomy. Transforming growth factor-β (TGF-β) is considered to be a key molecule for the transformation of keratocytes into myofibroblasts, and the subsequent production of extracellular matrix.[13] As tranilast has an inhibitory effect on the activity of TGF-β, such as the production of extracellular matrix,[14] we speculate that the mechanism for the inhibition of haze might be due to the reduction of the TGF-β-evoked transformation of keratocytes, production of extracellular matrix, as well as the proliferation of keratocytes in the injured stroma.

On the other hand, the subepithelial haze developing after PTK or PRK is considered to be a non-specific scar,[2,3] consisting of extracellular matrix,

Jpn J Ophthalmol
Vol 46: 488–495, 2002

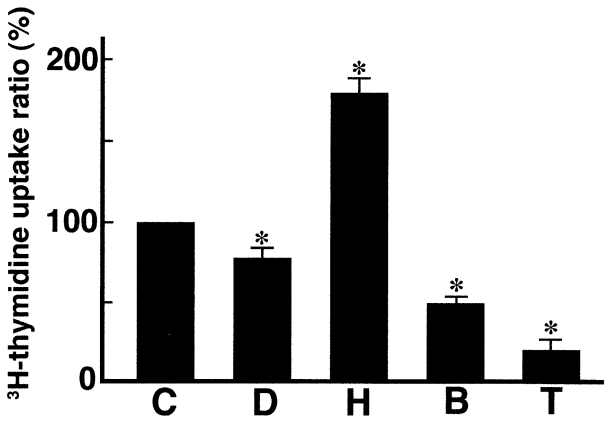

Figure 7. ^{3}H-thymidine incorporation in keratocytes 2 weeks after keratectomy. The number of ^{3}H-thymidine incorporating keratocytes was counted in each group, and standardized by the length of the sectioned tissue. Data in Control group (C) was taken as 100%. Data are shown as mean ± SEM values (obtained from five samples). The number of keratocytes incorporating ^{3}H-thymidine was significantly increased in Hyaluronic acid group (H) compared with Control group (C), and was significantly decreased in Tranilast (T), Betamethasone (B) and Diclofenac (D) groups ($*P < .01$ vs. control).

large molecular weight proteoglycans and collagen types III and IV.[6,15,16] These lines of evidence imply that tranilast may be useful for the prevention of subepithelial haze not only after mechanical keratectomy but also after PTK or PRK. Although there are studies demonstrating the effectiveness of tranilast in suppressing subepithelial haze after PRK in humans and animals,[9,10] histological studies are lacking. In this study, we verified that tranilast can inhibit keratocyte proliferation and collagen fiber production histologically as well.

Steroids are potent anti-inflammatory drugs that retard the process of wound healing by inhibiting DNA and protein synthesis. There are a number of clinical studies indicating the usefulness of steroids in suppressing subepithelial haze after PRK in humans.[17,18] Our study demonstrated that betamethasone suppresses the proliferation of keratocytes and the production of collagen fibers. However, steroid treatment for corneal haze remains controversial, due to several side effects. Moreover, it is also worth mentioning that steroids are not always effective in preventing corneal haze in the human eye, even if proven effective in many animal studies.[19,20]

Hyaluronic acid possesses a variety of biological activities. It keeps the corneal surface moist, acts as a viscoelastic substance, and promotes corneal wound healing.[21,22] In the present study, the corneal epithelium regenerated within 2 weeks in all groups. Close examination of the epithelium, however, revealed a difference in epithelial cell regeneration patterns. The epithelial layers were well regenerated in the Hyaluronic acid group, while those in the Betamethasone and Diclofenac groups were relatively thinner, suggesting a possible delay in epithelial cell regeneration in these groups. Our results also show that hyaluronic acid promoted the proliferation of keratocytes, which is consistent with previous studies.[23] Surprisingly, there was no abnormal accumulation of keratocytes in the subepithelial area, despite augmentation of keratocyte proliferation. These findings suggest that hyaluronic acid may promote the physiologic wound healing process in the corneal stroma and that the early regeneration of corneal epithelium might be a fundamental mechanism for the inhibition of corneal haze. Subepithelial haze is known to occur less frequently in laser in situ keratomileusis (LASIK), a procedure that maintains an intact epithelium, than in PRK, a procedure that completely removes the epithelium.[24] In fact, it has been suggested that the presence of an intact epithelium may affect the process of stromal wound healing and the development of subepithelial haze.[25]

Diclofenac exhibits an anti-inflammatory effect by inhibiting cyclooxygenase activity. It is used after ocular surgery for suppressing inflammation and pain. Diclofenac, as well as steroids, is reported to inhibit the proliferation of keratocytes in vitro. In this study, however, wound healing was retarded and corneal haze became more severe in the Diclofenac group. In this group, the injured area was accompanied by edema, neovascularization, inflammatory cell infiltration, and retrocorneal membrane formation. As the origin of the retrocorneal membrane is thought to be from the corneal endothelial cells, keratocytes or fibroblasts from the anterior chamber angle,[26] we speculate that the tissue damage in the Diclofenac group was so severe that it consequently stimulated migration of these cells into the injured corneal stroma. Although diclofenac has been used to reduce pain after corneal surgery,[27] several studies have demonstrated that diclofenac retards corneal wound healing and does not inhibit polymorphonuclear leukocyte infiltration or corneal haze formation after surgery.[28] Polymorphonuclear leukocytes have been shown to be responsible for the formation of the retrocorneal membrane through the action of basic fibroblast growth factor.[29] Diclofenac has been reported to inhibit epithelial regeneration. Our study demonstrated that epithelial regeneration was retarded in the Diclofenac group. Based on our re-

sults, the retardation in regeneration of the epithelium might have caused the severe inflammation and resulted in the development of stromal haze. In the case of steroids, which also retard regeneration of the epithelium, we speculate that corneal haze is inhibited because of the direct and potent anti-inflammatory action of the steroids.

In conclusion, this study demonstrated that tranilast and betamethasone inhibit the formation of stromal haze by inhibiting the proliferation of keratocytes and the production of extracellular matrix in the corneal stroma. Hyaluronic acid may inhibit stromal haze by promoting physiologic wound healing.

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