Inhibition of Corneal Inflammation by the Topical Use of Ras Farnesyltransferase Inhibitors: Selective Inhibition of Macrophage Localization

Koh-bei Sonoda, Taiji Sakamoto, Hiroshi Yoshikawa, Satomi Ashizuka, Yuji Oshshima, Kenji Kishibara, Kikuo Nomoto, Tatsuro Ishibashi, and Hajime Inomata

PURPOSE. Ras farnesyltransferase inhibitors are known to block the membrane translocalization of oncogenic Ras protein. They inhibit the cytoplasmic mitogen-activated protein kinase signaling cascade related to Ras protein. Thus far, Ras farnesyltransferase inhibitors have been exclusively regarded with the anticancer drugs. The object of this study was to elucidate the role of Ras farnesyltransferase inhibitors on the corneal opacity induced by an inflammatory stimulus.

METHODS. We used a cauterization-induced corneal inflammation model. The central corneas of BALB/c mice were cauterized with silver nitrate (1 mm in diameter). Ras farnesyltransferase inhibitors, either manumycin or gliotoxin eye drops (each drug dissolved in balanced salt solution [BSS] at concentrations of 1 mM), were topically delivered to the cauterized cornea every 8 hours; BSS eye drops were used as a control. Clinical signs such as corneal edema, opacity, and corneal neovascularization, which are major causes of visual disturbance, were then examined 96 hours after the cauterization. The corneal edema and opacity were clinically scored under a stereoscopic microscope. The corneal neovascularization was evaluated by the length of the blood vessels from the limbus and the sum of extension central angle of vascularized limbus. Furthermore, the corneas were examined histologically, and the phenotypes of the cornea-infiltrating cells were analyzed by flow cytometry.

RESULTS. The control corneas showed prominent edema, neovascularization, and opacity. Histologic analysis revealed corneal epithelial and endothelial cell loss and a large amount of inflammatory cell infiltration into the corneal stroma. Flow cytometric analysis revealed that most of the infiltrating cells were neutrophils and macrophages. In contrast, the degree of corneal edema, neovascularization, and opacity was significantly less in the manumycin- or gliotoxin-treated corneas than in the control corneas. Histologically, the manumycin- and gliotoxin-treated corneas showed minimum edema and good epithelialization. Flow cytometric analysis showed corneal infiltration of macrophages to be selectively and clearly inhibited. Neither manumycin nor gliotoxin produced any side effects in the noncauterized normal cornea either clinically or histologically.

CONCLUSIONS. Ras proteins play an important role in cauterization-induced corneal inflammation and the opacity it induces. Ras farnesyltransferase inhibitors thus have a great potential for improving the treatment of corneal opacity induced by a corneal inflammatory stimulus. (Invest Ophthalmol Vis Sci. 1998;39:2245-2251)

Corneal inflammation and its related visual disturbance are important and common clinical events in alkali-burn, herpetic keratitis, rejection of corneal grafts, and related conditions.

Inflammatory cell infiltration, edema, and neovascularization in the injured cornea may be associated with irreversible opacification and loss of vision.1

Ras proteins are known to be key molecules for initiating activation of the intracellular protein kinase cascade. Oncogenic Ras proteins constitutively activate the mitogen-activated protein kinase signaling cascade, thereby sending an uninterrupted growth signal.2 Ras proteins must be translocalized to the plasma membrane to function,3 and Ras farnesyltransferase (FTase) is also required for Ras membrane association. This membrane translocalization is facilitated by posttranslational modifications occurring at the C terminus of the Ras protein. The first step in a series of modifications is farnesylation of a conserved cysteine residue positioned four amino acids from the C terminus. After farnesylation, the three C-terminal amino acids are proteolytically removed, and then the newly formed farnesylcysteine residue is methylated.4

The importance of farnesylation to Ras transforming activity has promoted the development of FTase inhibitors that may have chemotherapeutic potential, especially against tumors.5 Inhibition of Ras FTase would not perturb other elements of...
UCF1-C had the strongest Ras FTase inhibitory activity among the mevalonate pathway and would be expected to be an effective antagonist of Ras function.

Thus far, a relationship between a Ras-mediated intracellular signal and corneal inflammation has not been demonstrated. Corneal inflammation might be mediated by such complex cellular events, for example, as cell migration, proliferation, and the production of cytokines. An alternative possibility is that a Ras-mediated intracellular signal might play a pivotal role in corneal inflammation. To elucidate this hypothesis, we used the cauterization-induced corneal inflammation model in mice.

In the present experiments, we used two different drugs that possess Ras FTase inhibitory activity. Manumycin was originally identified as an antibiotic. Recently, Hara et al. purified UCF1-A, -B, and -C, which are farnesyl diphosphate analogues identified by microbial screening. They found that UCF1-C had the strongest Ras FTase inhibitory activity among them, and they also found that UCF1-C was the same molecule that had previously been identified as manumycin. Gliotoxin had been identified as a fungal metabolite originally. Van der Pyl et al. reported that gliotoxin had strong Ras FTase inhibitory activity by a direct FTase assay in vitro. Although manumycin and gliotoxin have the same Ras FTase inhibitory activity, they are derived from completely different origins and have different chemical structures, as shown in Figure 1. With these two different drugs it was possible to observe the effects of Ras FTase inhibitors on corneal inflammation.

In this article, the inhibitory effects of Ras FTase inhibitors on corneal inflammation are reported. The implications of these observations are also discussed.

**Materials and Methods**

**Mice**

Seven-week old female BALB/c mice were bred in specific pathogen-free conditions at Kyushu University and were used in all experiments. All treatment of the animals conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Drugs**

Manumycin (UCF1-C) was kindly provided by the Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). Purification of the drug has been described previously. Manumycin was dissolved in a 1 mM balanced salt solution (BSS PLUS; Alcon, Fort Worth, TX) containing 0.1% DMSO. This solution was thus used as manumycin eye drops. Gliotoxin was purchased from Sigma Chemical Co. (St. Louis, MO), and was dissolved in 1 mM BSS PLUS containing 0.1% DMSO. This solution was then used as gliotoxin eye drops. The chemical structures of both drugs are shown in Figure 1.

**Induction of Corneal Inflammation**

Corneal inflammation was induced as described previously with some modifications. Mice were anesthetized with 1.0 mg sodium pentobarbital in 0.2 ml PBS injected intraperitoneally. The right cornea of each mouse was cauterized with an application stick containing 4 g/ml silver nitrate. The cautery sticks we used were made by us. We sharpened the plastic stick so that the tip diameter would be 1 mm to allow clear marking. A marked grayish white patch of cauterized tissue 1 mm in diameter was produced at the center of the cornea. Next, manumycin or gliotoxin eye drops were delivered topically to the cauterized corneas every 8 hours. In other mice, BSS PLUS eye drops were used as a positive inflammation control.

**Evaluation of Corneal Edema, Neovascularization, and Opacity**

After 96 hours, corneal edema, neovascularization, and opacity were evaluated by stereoscopic microscopy and slit lamp in a double-masked fashion. The magnification of observation ranged from ×15 to ×25. Indices of corneal edema and opacity were scored on a scale from 0 to +4 according to the severity of the lesions. For edema the scoring was as follows: 0, a completely normal cornea; +1, no evidence of any ongoing edema, the corneal thickness was less than normal thickness; +2, mild edema, the corneal thickness was actually increased but was less than two times that of the normal cornea. Edema was limited to the cauterized area, and scoring was as follows: +3, severe edema, the corneal thickness was more than two times that of the normal cornea, edema was limited to the cauterized area; +4, extensive edema, severe edema extended to the whole cornea, and corneal perforation occurred in some cases. For opacity the scoring was as follows: 0, completely clear cornea; +1, slight opacity; +2, mild opacity, iris and lens were visible; +3, severe opacity, iris and lens were invisible, opacity was limited to the cauterized area; and +4, extensive opacity, severe opacity extended to the whole cornea. Corneal neovascularization was evaluated by length and extension according to modifications of the method of Kenyon et al. Neovascularization in this model occurred in particular areas of the limbal vascular plexus. In these areas, neovascularized contiguous limbal circumferential zones were formed. In highly damaged corneas, neovascularization often occurred in the total limbal vascular plexus. To assess the vessel length, we measured the maximum vessel length from the limbs toward the cauterized area with a linear reticule within the total neovascular zones through a slit lamp. To assess the vessel extension, the central angle of each contiguous circumferential zone of neovascularization was measured with a 360° reticule; results are all expressed per cornea.

In the experiments described above, we examined the following groups: group 1, manumycin treatment only (without corneal cauterization); group 2, gliotoxin treatment only (without corneal cauterization); group 3, BSS PLUS-treated...
cauterized cornea (positive inflammation control); group 4, manumycin-treated cauterized cornea; and group 5, gliotoxin-treated cauterized cornea. In each group we examined 6 to 12 mice per experiment. Experiments were repeated four times and revealed comparable results. After clinical evaluation the mice were euthanatized, and their right eyes were removed. Three eyes were used for flow cytometry studies, and the remaining eyes were used for histologic evaluation.

Isolation and Counting of the Cornea-Infiltrating Cells
To examine the phenotypes of the inflammatory cells in the cornea, single cells were prepared according to the procedure for the isolation of hepatic lymphocytes, with some modifications. Three corneas were pooled, teased with scissors, and then shaken in medium supplemented with 0.5 mg/ml Collagenase type D (Boehringer-Mannheim, Mannheim, Germany) at 37°C for 40 minutes. As basic medium, we used RPMI 1640 (GIBCO Laboratories, Grand Island, NY) with 10% fetal calf serum (GIBCO), 10 mg/ml gentamycin, 5×10⁻⁵ M 2-mercaptoethanol, and 5 mg/ml HEPES buffer. The supernatants were collected, passed through cotton gauze, and washed three times; viable cells then were counted using trypan blue dye exclusion. It was necessary to use three corneas to get enough viable cells for flow-cytometric analysis in the process described above. A total of 12 corneas (4 individual pools) were examined per group.

Antibodies and Reagents
The following reagents were used for flow cytometric analysis. Phycoerythrin (PE)–conjugated anti-Mac-1 monoclonal antibody (mAb) (clone name, M1/70.15) was purchased from Caltag Laboratory (South San Francisco, CA). Fluorescein isothiocyanate (FITC)–conjugated anti-Gr-1 mAb (clone name, RB6-8C5) was purchased from Pharmingen (San Diego, CA). FITC-conjugated anti-F4/80 mAb (clone name, A3-1) was purchased from Caltag Laboratory. Biotin-conjugated anti-B220 mAb (clone name, RA3-6B2) was purchased from Pharmingen. Biotin-conjugated anti-Thy-1.2 mAb (clone name, 5A-8) was purchased from the Meiji Milk Product Co. (Tokyo, Japan). Biotin-conjugated anti-pan-CD45 mAb (clone name, F1/2) was purchased from GIBCO BRL (Gaithersburg, MD). Streptavidin-Red613 was purchased from GIBCO BRL. Propidium iodide was purchased from Sigma.

Flow Cytometry
Corneal infiltrating cells were adjusted to the designated concentrations and then were stained by single color with biotin-conjugated anti-Thy-1.2 mAb and anti-B220 mAb followed by streptavidin-Red613. The cells were also stained by three colors with PE-Mac-1 mAb, either FITC-Gr-1 or FITC-F4/80 mAb, and either propidium iodide or pan-CD45 mAb followed by streptavidin-Red613. Both macrophages and neutrophils have been reported to be Mac-1 surface molecule-positive. 11 F4/80 mAb stained macrophages,12 and Gr-1 mAb stained neutrophils.13 Flow cytometry was performed with a fluorescence-activated cell sorter (Calibur; Becton-Dickinson, San Jose, CA). The numbers of cornea-infiltrating neutrophils and macrophages were calculated from the percentage of each population in the gate of propidium iodide-negative staining viable cells and the precounted total number of viable cells using trypan blue dye exclusion.

Histologic Examination
Some of the right eyes that had been removed were fixed in 10% phosphate-buffered formalin and then embedded in paraffin. Next, 4-μm sections were prepared and stained with hematoxylin and eosin.

Statistics
The data were statistically evaluated by the two-tailed Student’s t-test. All values of P < 0.05 were considered significant.

RESULTS
Corneal Edema, Neovascularization, and Opacity with Ras Farnesyltransferase Inhibitors
Corneal edema, neovascularization, and opacity were observed daily after cauterization. Ninety six hours later, the BSS-treated control corneas showed prominent edema, vessel involvement toward the cauterization scar, and central opacity (Fig. 2A). In contrast, manumycin- and gliotoxin-treated corneas showed a minimum involvement of vessels and almost clear corneas (Figs. 2B, 2C).

We graded the level of corneal edema, opacity, and neovascularization clinically as described in the Materials and Methods section. Typical results from one experiment are shown in Figure 3. Experiments were performed four times and revealed comparable results. As shown in Figures 3A and 3B, manumycin- and gliotoxin-treated corneas showed significantly less edema and opacity than the control corneas. Furthermore, neovascularization was also significantly inhibited in the manumycin- and gliotoxin-treated corneas compared with control corneas in regard to vessel length and vessel extension (Figs. 3C, 3D).

In addition, the naive corneas treated with manumycin and gliotoxin showed no edema or opacity (Figs. 3A, 3B) but showed minimal neovascularization (Fig. 3D). However, this neovascularization occurred in a very restricted area (Fig. 3C), and these slight vessels are often observed even in naive corneas. There were no significant changes when manumycin- and gliotoxin-treated corneas were compared with the naive corneas (data not shown). On the basis of these findings, we thus concluded that neither drug had a clinically detrimental effect on the normal corneas.

Modulation of Histologic Features by the Ras Farnesyltransferase Inhibitor
Figure 4 shows the histologic sections of corneas in each treatment at 96 hours after cauterization. The BSS-treated control corneas showed prominent edema (Fig. 4A) and an infiltration of inflammatory cells (Fig. 4B). The corneal thickness was about two times that of the normal cornea due to edema, and some corneas were also perforated. The corneal endothelial cells and epithelial cells were lost at the site of corneal edema (Fig. 4A). Inflammatory cells were also involved in the anterior chamber (Fig. 4A).

In contrast, manumycin-treated corneas and gliotoxin-treated corneas both showed minimal edema, and their thicknesses were less than that of the normal corneas. At the same time, good epithelialization was observed (Figs. 4C, 4D, 4E, 4F). The number of cells infiltrating the cornea were markedly decreased (Figs. 4D, 4F). There was also no evidence of inflammation in the anterior chamber.
FIGURE 2. A photograph of the corneas in each treatment on day 4. The corneas were cauterized with an application stick containing silver nitrate. Next, manumycin or gliotoxin eye drops were topically delivered to cauterized corneas every 8 hours. (A) BSS PLUS-treated control cornea (positive control). (The opacity caused by silver nitrate application is indicated by an arrow. Note that the pupil is obscured.) (B) Manumycin-treated cornea. (C) Gliotoxin-treated cornea (note that the pupil is visible in B and C). BSS, balanced salt solution.

The corneas treated with manumycin or gliotoxin alone (without corneal cauterization) showed no histologic changes (Figs. 4G, 4H) compared with the naive cornea (Fig. 4I). We thus confirmed that neither drug had a detrimental effect on the normal cornea histologically.

Modulation of Phenotype of the Cornea-Infiltrating Cells by Ras Farnesyltransferase Inhibitor Treatment

To elucidate the phenotype of cornea-infiltrating cells, single cells were prepared from corneas. Although we pooled three corneas, we could not obtain reliable counts of viable cells from the manumycin treatment-only group (without corneal cauterization) and gliotoxin treatment-only group (without corneal cauterization).

Figure 5A shows the total number of viable cells adjusted per cornea for each treatment. Compared with the control corneas, the number of infiltrating cells significantly decreased in the manumycin- and gliotoxin-treated cornea groups.

The cellular events that occur in corneal cauterization have been reported on the basis of an immunohistochemical assay. Neutrophils and macrophages infiltrate the cauterized cornea at 96 hours after cauterization. T cells, B cells, and mast cells are rarely seen during the entire inflammatory process. Our flow cytometry data were almost identical. As shown in Figure 6, most of the infiltrating cells in the control corneas were neutrophils and macrophages. Viable cells that did not stain for anti-Mac-1 mAb were probably isolated resident corneal cells and not bone marrow-derived cells, because they were not stained by anti-pan CD45 mAb, a common marker of...
Ras FTase Inhibitors Reduce Corneal Opacity

FIGURE 4. Histologic sections of the cauterized cornea: 96 hours after cauterization, the eyes were removed and then examined histologically. (A, B) Sections of balanced salt solution (BSS)-treated cornea (positive control)(A, ×30; B, ×75). (C, D) Sections of the manumycin-treated cornea (C, ×30; D, ×75). (E, F) Sections of the gliotoxin-treated cornea (E, ×30; F, ×75). (G) Section of the manumycin-treated cornea without corneal cauterization (×75). (H) Section of the gliotoxin-treated cornea without corneal cauterization (×75). (I) Section of naive cornea (×75).

Bone marrow–derived cells (data not shown). The proportion of anti–Thy-1 mAb–positive T cells and anti-B220 mAb–positive B cells was less than 1% of the total viable cells (data not shown). Surprisingly, macrophage infiltration was completely blocked in the manumycin- and gliotoxin-treated corneas. The proportion of neutrophils in the cornea-infiltrating cells was almost the same in these three groups (Fig. 5).

We next calculated the total number of infiltrating neutrophils and macrophages, adjusted per cornea in each treatment. In the manumycin-treated corneas, the number of neutrophils did not significantly decrease. In contrast, the number of infiltrating macrophages significantly decreased in the manumycin-treated cornea (P < 0.01). In gliotoxin-treated corneas, the number of infiltrating neutrophils and macrophages significantly decreased (Fig. 6).

DISCUSSION

In this investigation, we found that Ras FTase inhibitors have a great potential to inhibit corneal inflammation that is induced by cauterization. They had no side effects on normal corneas. These drugs reduced corneal edema, neovascularization, and opacity. Moreover, they also selectively inhibited macrophage localization at the cornea. Ras FTase inhibitors have been noted to be anti-cancer drugs because the ras gene has been regarded as one of the most important oncogenes.\cite{3,15} Manumycin is known to inhibit Ki-ras-transformed fibrosarcoma\cite{6} and the ras-activated human hepatoma cell line.\cite{16} In this article, we thus report another biological effect of Ras FTase inhibitors.

The most important question is the mechanism of reduction of corneal inflammation by the topical use of Ras FTase inhibitors. As shown in this article, we would like to emphasize that Ras FTase inhibitors selectively inhibited macrophage localization in the cauterized cornea. Macrophages have been implicated as mediators of corneal inflammation and the neovascular process in several studies.\cite{17,18} Macrophages produce various inflammatory cytokines and angiogenic factors such...
as tumor necrosis factor-α (TNF-α), basic fibroblast growth factor, insulin-like growth factor-1, interleukin-1 (IL-1), and vascular endothelial growth factor. 18 Van der Veen et al. 19 demonstrated that depletion of macrophages by the subconjunctival injection of dichloromethylene diphosphonate (CL2MDP)-containing liposomes could reduce neovascularization in the rat corneal allograft model.

The precise mechanisms by which Ras FTase inhibitors inhibit macrophage localization in the cauterized cornea have yet to be elucidated, but there are some possible explanations. One possibility is that Ras FTase inhibitors inhibit the production of macrophage chemoattractants. Corneal kerocytes have been reported to produce such β-chemokines (C-C chemokines) as chemokines regulated on activation, normal T-cell–expressed and –secreted (RANTES) protein, and monocyte chemotactic protein-1 after exposure to proinflammatory cytokines IL-1-α and TNF-α. 20 In general, the α-chemokines (C-X-C chemokines) are potent chemoattractants for neutrophils but not monocytes, whereas β-chemokines act on monocytes but have little effect on neutrophils. 21 Ras FTase inhibitors may operate corneal resident cells like kerocytes to prevent the production of macrophage chemoattractants like β-chemokines but do not prevent the production of neutrophil chemoattractants like α-chemokines.

Another possibility is that Ras FTase inhibitors have a direct effect on macrophages. Many adhesion molecules are expressed on the surface of macrophages, and some of them might be essential for the localization of macrophages in the cauterized cornea. Ras FTase inhibitors may directly inhibit the expression of adhesion molecules and thus prevent macrophages from localizing in the cornea.

Moreover, Ras FTase inhibitors may induce cell death, such as apoptosis, in infiltrating macrophages. In particular, gliotoxin has been reported to induce apoptosis in a variety of cell types, including macrophages. 22 This mechanism might explain the fact that gliotoxin inhibited cell localization in the cornea more significantly than manumycin as shown in Figure 6. Thus, through one or a combination of the above-mentioned possibilities, Ras FTase inhibitors tend to inhibit macrophage localization.

There also remains a possibility that Ras FTase inhibitors inhibit some other factors that promote corneal opacity independent of macrophage function. One of the principal factors is neovascularization itself, and the relationship between the function of vascular endothelial cells and Ras FTase inhibitors might be important also. Arbiser et al. recently demonstrated that the introduction of activated H-ras into immortalized endothelial cells stimulated angiogenesis. 23 The Ras FTase inhibitor might directly inhibit the migration, localization, and proliferation of vascular endothelial cells in the cauterized cornea. As a result, it might reduce neovascularization and subsequently induce irreversible corneal opacity. Further investigations will be needed to elucidate the effects of Ras FTase inhibitors on the process of corneal neovascularization.

Based on the findings noted above, Ras FTase inhibitors are thus considered to have a great therapeutic potential against corneal inflammation and the related blindness induced by various corneal diseases.

Acknowledgments

The authors thank Mari Imamura for her help in preparing the histologic sections; Mitsunobu Hara (Kiyowa Hakko Kogyo Co., Ltd., Tokyo, Japan), Hiroki Sanui, and Masao Uehara for assisting with this study, and Joan Stein-Streilein and J. Wayne Streilein (Schepens Eye Research Institute, Harvard Medical School, Boston, Massachusetts) for their careful reading and helpful comments on our manuscript.

References

Ras FTase Inhibitors Reduce Corneal Opacity


ANNOUNCEMENTS

Association for Ocular Pharmacology and Therapeutics

The 4th Annual AOPT Meeting will be held January 28 to 31, 1999, at The Marriott, Irvine, CA. Emphasis of the meeting is current advances in pharmacology as they relate to the eye and vision. For further information contact: Achim Krauss, Allergan, Inc., 2525 Dupont Dr., Irvine, CA 92713 (tel: 714-246-4842, fax: 714-246-5578; e-mail: krauss_achim@allergan.com).