Inhibition by Tranilast of the Cytokine-Induced Expression of Chemokines and the Adhesion Molecule VCAM-1 in Human Corneal Fibroblasts

Tadafumi Adachi, Ken Fukuda, Yukiko Kondo, and Teruo Nisibida

Purpose. The synthesis of chemokines and adhesion molecules by corneal fibroblasts contributes to the development of corneal lesions in severe ocular allergy. The effects of the antiallergy drug tranilast on the expression of such molecules were examined in human corneal fibroblasts.

Methods. The release of chemokines into culture supernatants and the expression of vascular endothelial cell adhesion molecule (VCAM)-1 on the cell surface were determined with enzyme-linked immunosorbent assays. The intracellular abundance of mRNAs was quantitated by reverse transcription and real-time polymerase chain reaction analysis. The phosphorylation of signaling proteins was examined by immunoblot analysis.

Results. Tranilast inhibited the release of the chemokines eotaxin-1 and TARC and the surface expression of VCAM-1, induced by the combination of TNF-α and IL-4 in corneal fibroblasts. Dexamethasone, but not cyclosporine A or tacrolimus, mimicked these effects of tranilast. Tranilast also inhibited the cytokine-induced upregulation of eotaxin-1 and TARC mRNAs in corneal fibroblasts. Tranilast inhibited the cytokine-induced phosphorylation of the NF-κB inhibitor IκBα and of mitogen-activated protein kinases (ERK, JNK, p38), without affecting that of STAT6, in corneal fibroblasts.

Conclusions. Inhibition by tranilast of the cytokine-induced expression of eotaxin-1, TARC, and VCAM-1 in human corneal fibroblasts suggests that this drug might prove effective for treatment of the corneal manifestations of ocular allergic inflammation by targeting corneal fibroblasts directly. (Invest Ophthalmol Vis Sci. 2010;51:3954–3960) DOI:10.1167/iovs.09-4161

Verbal keratoconjunctivitis (VKC) is a severe and chronic ocular allergy associated with pronounced infiltration into the conjunctiva of inflammatory and immune cells such as eosinophils, mast cells, and T helper 2 (Th2) lymphocytes. A variety of cytokines, chemokines, and enzymes secreted by these infiltrated cells contribute to the clinical characteristics of VKC, including giant papillae and corneal lesions.1,2 Among these bioactive substances, the proinflammatory cytokine tumor necrosis factor (TNF)-α and the Th2 cytokine interleukin (IL)-4, which are released from mast cells and Th2 cells, respectively, play important roles in triggering allergic inflammation in the conjunctiva by activating resident cells. Unlike other types of allergic conjunctivitis, VKC affects the cornea and threatens visual acuity. Major signs and symptoms of allergic diseases of the conjunctiva include itching and swelling of the tissue, tearing, and ocular discomfort. However, in addition to these manifestations of ocular allergy, VKC is associated with corneal lesions such as superficial punctate keratopathy and corneal erosion, ulcer, and plaque.3 Although numerous anti-allergy drugs, including antihistamines, mast cell stabilizers, and immunosuppressants (cyclosporine A, tacrolimus), have been found to be effective for the treatment of VKC, none of these agents target the associated corneal lesions. Such lesions are thus often not prevented by, or are resistant to, these antiallergy drugs,3 and steroids are the only available choice for their treatment. The efficacy of steroids is likely attributable to effects on multiple cell types, including mast cells, T cells, eosinophils, and fibroblasts, but these drugs also have serious side effects.

The pathogenesis of corneal lesions in VKC remains unclear, though eosinophils are implicated in this process. The number of eosinophils in tear fluid thus correlates with the severity of corneal complications in VKC.4 Eosinophil granule components, such as major basic protein and eosinophil cationic protein, have also been detected in corneal ulcer and plaque in patients with VKC.5–7 These proteins inhibit corneal epithelial wound healing in organ culture and adversely affect the viability and morphology of human corneal epithelial cells in vitro.8,9 In addition, eosinophils express matrix metalloproteinase (MMP)-9, which degrades epithelial basement membrane components such as collagen type IV and laminin, and immunostaining for MMP-9 has been found to be increased in conjunctival eosinophils and in the corneal stroma at the base of ulcers in patients with VKC.5,10 Furthermore, the level of pro-MMP-9 in tear fluid of patients with VKC correlates with the eosinophil count, and MMP-9 activity in tear fluid of such persons correlates with the severity of corneal involvement.5,11 These various observations thus suggest that eosinophil-derived epitheliotoxins and basement membrane-degrading enzymes contribute, at least in part, to the development of corneal complications in VKC. Given that the concentration of eotaxin-1 in tear fluid also correlates with the severity of corneal lesions in VKC patients, this chemokine may induce the migration of eosinophils toward the cornea and thereby contribute to corneal complications.4 Eotaxin-1 is therefore a potential therapeutic target for corneal complications in VKC.

We and others12–14 have shown that TNF-α and IL-4 stimulate the synthesis of eotaxin-1 (CCL11) in corneal or conjunctival fibroblasts, but not in corneal epithelial cells. We have also shown that the combination of TNF-α and IL-4 induces a synergistic increase in the expression by corneal fibroblasts, but not by corneal epithelial cells, of thymus- and activation-regulated chemokine (TARC, or CCL17)15,16 and vascular en-
dothelial cell adhesion molecule-1 (VCAM-1),\textsuperscript{17} which together mediate the infiltration and activation of eosinophils and Th2 cells. Corneal epithelial cells thus do not express chemokines in response to Th2 cytokines but rather likely act as a physical and chemical barrier to prevent the penetration of inflammatory mediators into the stroma. Mechanical compression and contact as well as inflammatory mediators such as TNF-α and mast cell-derived chymase may disrupt the barrier function of corneal epithelial cells during allergic inflammation.\textsuperscript{18,19} Indeed, corneal epithelial barrier function is impaired in persons with atopic blepharoconjunctivitis.\textsuperscript{20} In addition, we have recently shown that the conjunctiva and cornea interact with each other in a rat model of ocular allergy, with loss of the barrier function of the corneal epithelium exacerbating conjunctival inflammation and conjunctival inflammation inhibiting corneal epithelial wound healing, suggestive of the operation of a vicious circle.\textsuperscript{21} In contrast, fibroblasts may play a key role in the induction and amplification of ocular allergic inflammation and the consequent development of corneal disorders in persons with VKC.\textsuperscript{22,23} Fibroblasts or fibroblast-derived factors thus represent potential targets for the development of new therapeutic agents for the treatment of corneal disorders associated with VKC.

Tranilast, or N-(3,4-dimethoxycinnamoyl) anthranilic acid, is an antiallergy drug that is administered orally or in eyedrops for the treatment of allergic asthma, atopic dermatitis, allergic rhinitis, and allergic conjunctivitis. As a mast cell stabilizer, tranilast inhibits the release of chemical mediators from mast cells and the allergy-associated increase in vascular permeability.\textsuperscript{24–25} However, it also inhibits the secretion of cytokines such as interleukins and transforming growth factor-β1, cell proliferation, and collagen synthesis in skin fibroblasts.\textsuperscript{26} It has thus also been applied clinically to the treatment of skin keloid and hypertrophic scarring, and its ability to target fibroblasts has been investigated in relation to the treatment of eye conditions such as proliferative vitreoretinopathy.\textsuperscript{27–28} corneal haze after photorefractive keratectomy,\textsuperscript{29} pterygium,\textsuperscript{30} fibrosis of filtering blebs,\textsuperscript{31} and posterior capsule opacification.\textsuperscript{32} In addition, tranilast was found to inhibit the synthesis of chemokines by various cell types,\textsuperscript{33–35} suggesting that the suppressive effect of tranilast on allergic inflammation might be mediated in part by the inhibition of chemokine or adhesion molecule expression by fibroblasts.

We have now investigated whether tranilast might affect the inflammatory response of corneal fibroblasts and might therefore prove effective for the treatment of corneal lesions associated with VKC. We examined the possible effects of this drug on the expression of the chemokines eotaxin-1 and TARC and of the adhesion molecule VCAM-1 induced by the combination of TNF-α and IL-4 in these cells.

Materials and Methods

Materials

Eagle’s minimum essential medium (MEM), phosphate-buffered saline (PBS), fetal bovine serum, and trypsin-EDTA were obtained from Invitrogen-Gibco (Grand Island, NY), and 24- and 96-well culture plates as well as 60-mm culture dishes were from Corning Glass (Corning, NY). Tranilast was kindly provided by Kissei Pharmaceutical Co. Ltd. (Tokyo, Japan), and dexamethasone, cyclosporine A, and tacrolimus were obtained from Sigma-Aldrich (St. Louis, MO). Recombinant human TNF-α and IL-4, as well as paired antibodies for human eotaxin-1 and TARC enzyme-linked immunosorbent assays (ELISAs), were obtained from R&D Systems (Minneapolis, MN). Mouse monoclonal antibodies to V-CAM-1 were from BD PharMingen (San Diego, CA). Mouse monoclonal antibodies to human phosphorylated IκBα and p38 mitogen-activated protein kinase (MAPK) and rabbit polyclonal antibodies to human extracellular signal-regulated kinase (ERK), phosphorylated ERK, c-Jun NH₂-terminal kinase (JNK), phosphorylated JNK, phosphorylated p38 MAPK, signal transducer and activator of transcription 6 (STAT6), and phosphorylated STAT6 were from Cell Signaling (Beverly, MA). All media and reagents used for cell culture were endotoxin minimized.

Isolation and Culture of Human Corneal Fibroblasts

Human corneas were obtained from Mid-America Transplant Service (St. Louis, MO), Northwest Lions Eye Bank (Seattle, WA), and The Eye Bank of Wisconsin (Madison, WI). Donors were white males and females ranging in age from 4 to 65 years. The center of each donor cornea was punched out for corneal transplantation surgery, and the remaining rim of tissue was used for the present experiments. The human material was used in strict accordance with the basic principles of the Declaration of Helsinki. Corneal fibroblasts were prepared and cultured as described previously.\textsuperscript{12,15} Tissue from each cornea was digested separately with collagenase to provide a suspension of corneal fibroblasts, and the cells from each cornea were cultured independently in MEM supplemented with 10% fetal bovine serum in 60-mm dishes until they had achieved approximately 90% confluence. They were used for the present experiments after four to seven passages. As described previously,\textsuperscript{36} the purity of the cell cultures was judged on the basis both of the distinctive morphology of corneal fibroblasts and of their reactivity with antibodies to vimentin, cytokeratin, and α-smooth muscle actin in immunofluorescence analysis. All the cells used in the present study were positive for vimentin and negative for both cytokeratin and α-smooth muscle actin, suggesting that the cultures were not contaminated by epithelial cells and that they comprised fibroblasts rather than myofibroblasts.

ELISA for Chemokines

Corneal fibroblasts were cultured in 24-well plates until they achieved confluence, after which the culture medium was replaced with serum-free MEM for 1 day. The cells were then incubated first for 1 hour with or without tranilast (300 μM) in serum-free MEM and then for 48 hours in the additional absence or presence of TNF-α and IL-4 (each at 10 ng/mL). The medium was then collected and centrifuged at 120g for 5 minutes, and the resultant supernatant was frozen at −80°C for subsequent assay of chemokines. The concentrations of eotaxin-1 and TARC in the culture supernatants were determined by ELISA, as previously described,\textsuperscript{12,57} with measurement of absorbance at 450 nm, and they were normalized by expression as nanograms or picograms of chemokine per 1 × 10⁶ cells.

RT and Quantitative PCR Analysis

Total RNA was isolated from cells and subjected to reverse transcription (RT) with the use of a kit (Promega, Madison, WI). The abundance of eotaxin-1, TARC, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNAs was quantified by real-time polymerase chain reaction (PCR) analysis with an automated PCR system (LightCycler; Roche Molecular Biochemicals, Indianapolis, IN), as described previously.\textsuperscript{13,36} Whole-Cell ELISA for VCAM-1

For detection of VCAM-1, a whole-cell ELISA was performed as previously described.\textsuperscript{17,59,60} Corneal fibroblasts (5 × 10⁵ cells/well) were cultured in 96-well, flat-bottomed microtiter plates for 72 hours, after which the culture medium was changed to serum-free MEM and the cells were incubated for an additional 24 hours. The cells were then incubated in MEM supplemented with various concentrations of tranilast for 1 hour before incubation in the additional absence or presence of IL-4 and TNF-α (each at 10 ng/mL) for 24 hours. After two washes with PBS, the cells were fixed for 15 minutes at room temperature with PBS containing 1% paraformaldehyde. They were then washed with PBS containing 0.1% bovine serum albumin (BSA), incubation protocol described previously,\textsuperscript{12,36} starting with a 1:1000 dilution of antibody followed by a 1:2000 dilution of a horseradish peroxidase-conjugated secondary antibody.
bated for 1 hour at 37°C with antibodies to VCAM-1 (1:10,000 dilution) in PBS containing 1% BSA, washed three times with PBS-BSA (1%), and incubated for 1 hour at 37°C with horseradish peroxidase-conjugated goat antibodies to mouse immunoglobulin G (Chemicon, Temecula, CA) in PBS-BSA (1%). After three washes with PBS-BSA (1%), the cells were incubated for 15 minutes at room temperature in the dark with 100 μL tetramethylbenzidine solution. The reaction was then terminated by the addition of 50 μL of 1 M H₂SO₄, and the absorbance of each well was determined at 450 nm with the use of a microplate reader (PowerWave XS; Bio-Tek Instruments, Winooski, VT).

**Statistical Analysis**

Data are presented as mean ± SEM and were analyzed with Dunnett's test, Scheffe’s multiple comparison test, or Student's unpaired t-test. *P* < 0.05 was considered statistically significant.

**RESULTS**

**Inhibition of Eotaxin-1 and TARC Release from Human Corneal Fibroblasts by Tranilast**

We first examined the release of the chemokine eotaxin-1 by corneal fibroblasts incubated for 48 hours with TNF-α (10 ng/mL), IL-4 (10 ng/mL), or the combination of TNF-α and IL-4. Consistent with our previous results, although incubation of corneal fibroblasts with TNF-α or IL-4 alone induced relatively small increases in eotaxin-1 release, exposure of the cells to both agents resulted in a pronounced increase in this parameter (Fig. 1A). Tranilast (300 μM) significantly inhibited eotaxin-1 release from these cells stimulated with TNF-α, IL-4, or both agents. Examination of the time course of eotaxin-1 release by corneal fibroblasts incubated for up to 72 hours with both TNF-α and IL-4 in the absence or presence of tranilast revealed that tranilast significantly inhibited the effect of these cytokines at 36 to 72 hours (Fig. 1B). The inhibitory effect of tranilast on eotaxin-1 release induced by TNF-α and IL-4 was also concentration dependent and was significant at concentrations of ≥10 μM and maximal at 300 μM (Fig. 1C). At the concentrations used in this study, tranilast did not manifest a cytotoxic effect on corneal fibroblasts, as revealed by the measurement of cell number or the release of lactate dehydrogenase (data not shown).

Consistent with our previous data, RT and real-time PCR analysis revealed that incubation of corneal fibroblasts with both TNF-α and IL-4 for 12 hours resulted in a 37-fold increase in the amount of eotaxin-1 mRNA (Fig. 2). This effect of the two cytokines was inhibited by approximately 60% in the presence of tranilast (300 μM), suggesting that the inhibitory effect of tranilast on eotaxin-1 release induced by the combination of TNF-α and IL-4 in corneal fibroblasts is mediated, at least in part, at the level of eotaxin-1 gene expression.

We next examined the effect of tranilast on the release by corneal fibroblasts of the chemokine TARC. As we showed previously, although TNF-α or IL-4 alone did not induce TARC release by corneal fibroblasts, the combination of both TNF-α and IL-4 elicited a marked increase in this parameter. This effect of TNF-α and IL-4 was inhibited by tranilast in a time- and concentration-dependent manner (Figs. 3A, 3B). Tranilast also inhibited by approximately 70% the increase in the abundance of TARC mRNA induced by TNF-α and IL-4 (Fig. 3C).

**Figure 1.** Effect of tranilast on cytokine-induced eotaxin-1 release by corneal fibroblasts. (A) Cells were incubated first for 1 hour with (closed bars) or without (open bars) tranilast (300 μM) and then for 48 hours in the additional absence or presence of TNF-α (10 ng/mL), IL-4 (10 ng/mL), or both cytokines, after which the amount of eotaxin-1 in the culture supernatants was determined. All data are expressed as nanograms of eotaxin-1 per 1 × 10⁶ cells and are mean ± SEM from four experiments. *P* < 0.01 (Scheffe’s test) versus the corresponding value for cells incubated without tranilast.

**Figure 2.** Effect of tranilast on the cytokine-induced increase in the abundance of eotaxin-1 mRNA in corneal fibroblasts. Cells were incubated first for 1 hour with (shaded bars) or without (open bars) tranilast (300 μM) and then for 12 hours in the additional absence or presence of TNF-α and IL-4 (each at 10 ng/mL). The amount of eotaxin-1 mRNA in the cells was then determined by RT and real-time PCR analysis. Data were normalized on the basis of the abundance of GAPDH mRNA, are expressed in arbitrary units, and represent the mean ± SEM of four experiments. *P* < 0.01 versus the corresponding value for cells incubated without cytokine. †P < 0.01 (Scheffe’s test) versus the corresponding value for cells incubated without tranilast.
We also examined the effects of other immunosuppressants used clinically for the treatment of severe ocular allergy on the release of eotaxin-1 and TARC by corneal fibroblasts. Dexamethasone (0.01–1.0 μM), but not cyclosporine A or tacrolimus (10–1000 ng/mL), significantly inhibited the release of both eotaxin-1 (Fig. 4) and TARC (data not shown) by corneal fibroblasts stimulated with the combination of TNF-α and IL-4.

**Inhibition of Surface Expression of VCAM-1 in Corneal Fibroblasts by Tranilast**

We examined the effect of tranilast on expression of the adhesion molecule VCAM-1 in corneal fibroblasts with the use of whole-cell ELISA. As we showed previously, exposure of corneal fibroblasts to the combination of TNF-α with IL-4 resulted in a marked increase in VCAM-1 expression at the cell surface. This upregulation of VCAM-1 expression by TNF-α and IL-4 was inhibited by tranilast in a concentration-dependent manner, with this inhibitory effect significant at concentrations of ≥100 μM (Fig. 5A). Dexamethasone, but not cyclosporine A or tacrolimus, also inhibited the stimulatory effect of TNF-α and IL-4 on VCAM-1 expression (Fig. 5B).

**Discussion**

We have shown here that tranilast inhibited the cytokine-induced expression of the chemokines eotaxin-1 and TARC and of the adhesion molecule VCAM-1 in human corneal fibroblasts. Eotaxin-1 and TARC function as key chemoattractants for eosinophils and Th2 cells, respectively, and VCAM-1 is an important adhesion molecule for both eosinophils and Th2 cells. Therefore, the anti-inflammatory effects of tranilast on these cell types suggest a possible role for this drug in the treatment of allergic ocular disease.
cells. Our results thus suggest that the clinical effects of tranilast might be attributable not only to its activity as a mast cell stabilizer but also to inhibition of late-phase allergic inflammation mediated at the level of chemokine and adhesion molecule expression by tissue resident fibroblasts.

We previously showed that TNF-α and either IL-4 or IL-13 induce synergistic increases in the expression of eotaxin-1, TARC, and VCAM-1 in corneal fibroblasts. The cytokine-induced synthesis of eotaxin-1 is regulated by MAPK, NF-κB, and STAT6 signaling pathways in various cell types. We have now examined the effects of tranilast on MAPK, NF-κB, and STAT6 signaling pathways in corneal fibroblasts. Tranilast inhibited the phosphorylation of ERK, JNK, p38 MAPK, and IκBα without affecting that of STAT6, induced by TNF-α and IL-4 in these cells. Consistent with our results, tranilast was previously shown to inhibit the cytokine-induced phosphorylation of JNK, IκBα, and p38 MAPK in other cell types. In addition, tranilast was previously shown to inhibit the TNF-α-induced, NF-κB-dependent surface expression of VCAM-1 and cytokine secretion in vascular endothelial cells by interfering with the association between NF-κB and cAMP response element–binding protein. Together, these various observations suggest that tranilast may block the activation of NF-κB and MAPK signaling pathways in corneal fibroblasts, resulting in inhibition of the expression of various chemokines, cytokines, and adhesion molecules.

In the present study, we focused on the inhibitory effects of tranilast on the expression of eotaxin-1, TARC, and VCAM-1 by corneal fibroblasts, given that these three molecules play central roles in the infiltration and activation of eosinophils and Th2 cells during allergic inflammation. In addition, however, we also found that tranilast inhibited the release of the cytokines or chemokines IL-6, G-CSF, MCP-1, MIP-1β, RANTES, IL-8, and IP-10 from corneal fibroblasts induced by TNF-α and IL-4 (unpublished data, 2008). Tranilast might thus have the potential to suppress the infiltration of various inflammatory cells, such as monocytes, neutrophils, and Th1 cells, in inflammatory conditions. Consistent with our results, tranilast was previously shown to inhibit the cytokine-induced secretion of IL-6, eotaxin-1, MCP-1, and IL-8 and the surface expression of VCAM-1 in various cell types.

We have shown that tranilast inhibited the expression of various inflammatory mediators in corneal fibroblasts, suggesting that this drug affects not only inflammatory cells but also corneal resident cells. Tranilast has been used clinically as a mast cell stabilizer for the treatment of allergic conjunctivitis.

**Figure 5.** Effect of tranilast on cytokine-induced VCAM-1 expression in corneal fibroblasts. (A) Cells were incubated first for 1 hour with the indicated concentrations of tranilast and then for 24 hours in the additional absence (open circles) or presence (closed circles) of TNF-α and IL-4 (each at 10 ng/mL each), after which the surface expression of VCAM-1 was determined by whole-cell ELISA. (B) Cells were incubated for 1 hour with or without tranilast (300 μM), dexamethasone (Dex, 1 μM), cyclosporine A (Cys A, 100 ng/mL), or tacrolimus (100 ng/mL) and then for 24 hours in the additional absence or presence of TNF-α and IL-4 (each at 10 ng/mL), after which the surface expression of VCAM-1 was determined. All data are expressed as a percentage of the value for cells incubated with TNF-α and IL-4 alone and represent the mean ± SEM of four experiments. *P < 0.05, **P < 0.01 (Dunnett’s test) versus the corresponding value for cells incubated with TNF-α and IL-4 alone.

**Figure 6.** Effects of tranilast on the cytokine-induced phosphorylation of IκBα, MAPKs, and STAT6 in corneal fibroblasts. Cells were incubated first for 1 hour with or without tranilast (300 μM) and then for 30 minutes in the additional absence or presence of TNF-α and IL-4 (each at 10 ng/mL), after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to total or phosphorylated (p) forms of ERK, p38 MAPK, JNK, IκBα, or STAT6. Data are representative of three independent experiments.
It has also been used for the treatment of skin keloid because of its antifibrotic effects in skin fibroblasts. In addition, we found that dexamethasone, but not cyclosporine A or tacrolimus, exhibited anti-inflammatory effects on corneal fibroblasts similar to those of tranilast. Steroids are potent anti-inflammatory drugs and are used for the treatment of various eye diseases including ocular allergy. Cyclosporine A and tacrolimus have also been shown to be effective for the treatment of severe ocular allergy conditions such as atopic keratoconjunctivitis and VKC. These two immunosuppressive drugs inhibit signaling by the phoshatase calcineurin and thereby block IL-2 gene transcription in T cells. These various observations suggest that tranilast and dexamethasone might suppress the function of both immune cells and tissue resident fibroblasts, whereas cyclosporine A and tacrolimus may affect only immune cells. In summary, we have shown that tranilast inhibited the expression of allergy-related chemokines and the adhesion molecule VCAM-1 by corneal fibroblasts. The uncontrolled overproduction of such mediators by fibroblasts of the eye may prevent resolution of allergic inflammation and result in tissue remodeling or destruction leading to the development of giant papillae and corneal disorders in VKC. Tranilast might, therefore, prove effective for the treatment of severe ocular allergy in part through its inhibitory actions on fibroblasts.

References


