Dry eye syndrome (DES) is one of the most common ocular disorders, affecting approximately 10% to 20% of the adult population in Western countries and Japan.1,2 Due to an increase in time spent using visual display terminals, along with a reduction of tear film due to the increased usage of air conditioners, and frequent use of contact lenses, the number of DES patients has increased steadily.1 Although the pathogenesis of DES remains unclear, various factors, such as aging, hormonal changes, environmental factors (noxious and infectious agents), inflammation, and autoimmune factors can play an important role because an increased level of tear osmolarity has been found to correlate with the condition's severity.1,9 In response to elevated tear osmolarity, cells rapidly uptake Na+ and other ions to maintain intracellular ionic strength, which in turn leads to cell-cycle arrest and the induction of apoptosis, resulting in lacrimal grand dysfunction and damage to the ocular surface. To overcome these disadvantages, an osmoprotective stress response is induced in cells in which nuclear factor of activated T-cells 5 (NFAT5; also known as tonicity enhancer binding protein) plays a central role.11 Although hyperosmolarity is a key pathological factor and also a good biomarker for the diagnosis of DES, given that the extent of increased tear osmolarity has been found to correlate with the condition’s severity,1,9 several alterations in tear fluid properties cause chronic ocular (both corneal and conjunctive) surface irritation and inflammation, resulting in ocular dryness and discomfort and visual dysfunction, which greatly reduce patient quality of life.

Elevated tear osmolarity is a key pathological factor and also a good biomarker for the diagnosis of DES.1,3 Various nonsteroidal anti-inflammatory drugs (NSAIDs), including diclofenac sodium (diclofenac), were identified during the screening procedure. These NSAIDs were able to suppress hyperosmolarity-induced apoptosis and cell growth arrest. In contrast, other NSAIDs, including bromfenac sodium (bromfenac), did not exert such a protective action. Treatment of cells with diclofenac, but not bromfenac, stimulated both the nuclear localization and expression of NFAT5 under hyperosmotic conditions. In the rat model for DES, topical administration of diclofenac (but not bromfenac) to eyes reduced corneal surface damage without affecting the volume of tear fluid.

**PURPOSE.** Dry eye syndrome (DES) is characterized by an increase in tear osmolarity and induction of the expression and nuclear localization of an osmoprotective transcription factor (nuclear factor of activated T-cells 5 [NFAT5]) that plays an important role in providing protection against hyperosmotic tears. In this study, we screened medicines already in clinical use with a view of finding compounds that protect cultured human corneal epithelial cells against hyperosmolarity-induced cell damage.

**METHODS.** Viable cell number was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method and cellular NFAT5 level was measured by immunoblotting. The rat model for DES was developed by removal of the lacrimal glands, with an assessment of corneal surface damage based on levels of fluorescein staining and epithelial apoptosis.

**RESULTS.** Some nonsteroidal anti-inflammatory drugs (NSAIDs), including diclofenac sodium (diclofenac), were identified during the screening procedure. These NSAIDs were able to suppress hyperosmolarity-induced apoptosis and cell growth arrest. In contrast, other NSAIDs, including bromfenac sodium (bromfenac), did not exert such a protective action. Treatment of cells with diclofenac, but not bromfenac, stimulated both the nuclear localization and expression of NFAT5 under hyperosmotic conditions. In the rat model for DES, topical administration of diclofenac (but not bromfenac) to eyes reduced corneal surface damage without affecting the volume of tear fluid.

**CONCLUSIONS.** Diclofenac appears to protect cells against hyperosmolarity-induced cell damage and NFAT5 would play an important role in this protective action. The findings reported here may also indicate that the topical administration of diclofenac to eyes may be therapeutically beneficial for DES patients.

Keywords: diclofenac, hyperosmolarity, nuclear factor of activated T-cells 5, dry eye syndromes
rapidly activates NFAT5, thereby enhancing the latter’s nuclear translocation,\(^\text{11,12}\) and it also gradually induces the expression of NFAT5.\(^\text{13}\) NFAT5 positively regulates the gene expression of various osmoprotective proteins.\(^\text{14}\) Among them, proteins responsible for the synthesis and transport of compatible small organic osmolytes (such as betaine/GABA transporter-1) especially play important roles, as they are able to suppress the increase in intracellular ionic strength by accumulating compatible small organic osmolytes in cells.\(^\text{15}\)

Patients with DES were previously treated with artificial tear supplementation, which provides only a temporary relief of symptoms. In addition to punctal occlusion and tear replacement therapy, clinical treatment for DES patients has been improved recently by the development of new types of drugs, such as anti-inflammatory agents, sodium hyaluronate, hormones, and secretagogues to stimulate mucin and tear fluid generation (such as rebamipide and diquafosol).\(^\text{1,16-19}\) However, these drugs have drawbacks, such as low efficacy, adverse effects, and the requirement of frequent instillation. Difficulties therefore remain for eye-care professionals to manage moderate-to-severely affected DES patients. Thus, the development of DES drugs with novel mechanisms of action is required.

Recent studies have revealed that hyperosmolarity-induced ocular epithelial cell apoptosis plays an important role in the pathogenesis of DES.\(^\text{20,21}\) Ocular epithelial cell apoptosis reduces the mucin layer, which in turn stimulates evaporative tear loss. To this end, accelerated ocular epithelial cell apoptosis was observed in DES patients and in animal models of DES.\(^\text{22-25}\) Furthermore, in line with an improvement of DES symptoms in response to treatment with cyclosporine A, a decrease in ocular epithelial cell apoptosis was observed in DES patients.\(^\text{24}\) Although compounds that protect ocular epithelial cells against hyperosmolarity may serve as good candidates for DES drugs, such compounds have not yet been identified.

In the present study, we used a library of approved medicines to screen for compounds that protect cultured human corneal epithelial (HCE) cells from hyperosmolarity-induced cell damage, with diclofenac sodium (diclofenac) identified as a potential candidate drug. Diclofenac is a commonly used nonsteroidal anti-inflammatory drug (NSAID). The findings from this study suggest that diclofenac suppresses hyperosmolarity-induced apoptosis and cell growth arrest in a process mediated both by the nuclear translocation of NFAT5 and induction of its expression. In a rat model for dry eye, topical administration of diclofenac to eyes reduced damage to the ocular surface and suppressed ocular epithelial cell apoptosis. These results suggest that the topical administration of diclofenac to eyes could serve as a good candidate therapy for DES patients.

### Materials and Methods

**Materials, Assay for Caspase Activity, Real-Time RT-PCR Analysis, BrdU Incorporation Assay, Immunoblotting Analysis, TdT-Mediated Biotinylated UTP Nick End Labeling (TUNEL), and Immunohistochemical Analyses**

Details are shown in the Supplementary Material.

**Cell Culture**

A human SV-40 immortalized corneal epithelial cell line (RCB 2280) was obtained from the Riken Bioresource Center Bank (Ibaraki, Japan) and used as HCE cells in this study. Human corneal epithelial cells were cultured in a mixture of Dulbecco’s modified Eagle’s medium and Nutrient Mixture F-12 medium, supplemented with 5% fetal bovine serum, 10 ng/mL epidermal growth factor, 1 μg/mL insulin, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere of 95% air with 5% CO\(_2\) at 37°C.

To examine the effect of NSAIDs on hyperosmolarity-dependent cell damage, cells (8 × 10\(^5\) cells/24-well plate or 1 × 10\(^3\) cells/100-mm dish) were exposed to hyperosmolarity by changing the entire bathing medium into that containing high concentration of each chemical (150 mM NaCl, 280 mM glucose or sorbitol; approximately 600 mOsm/L) and/or NSAID. Viable cell number was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method.\(^\text{26}\) Briefly, cells were incubated for 2 hours with MTT solution at a final concentration of 0.5 mg/mL. Isopropanol and hydrochloric acid were added to the culture medium at final concentrations of 50% and 20 mM, respectively. The optical density of each sample at 570 nm was determined spectrophotometrically by using a reference wavelength of 630 nm.

Determination of prostaglandin E\(_2\) (PGE\(_2\)) level in the culture media was done by enzyme immunoassay, as previously described.\(^\text{20}\)

### Analysis With the Rat Model for Dry Eye

All animal experiments were approved by the Laboratory Animal Care and Use Committee of Keio University. All studies adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Six-week-old male Sprague-Dawley rats were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). The rat model for dry eye was prepared by removing the lacrimal glands.\(^\text{27}\) Administration of NSAID to two eyes (three times daily) was done from 1 to 5 weeks after the removal of lacrimal glands. To estimate the degree of corneal surface damage at 5 weeks after the removal of lacrimal glands, 2 μL 0.05% fluorescein in PBS was instilled into the inferior conjunctival sac and the corneal surface was photographed. The fluorescein score was estimated as described previously.\(^\text{28}\) Briefly, the photographic image was divided into nine areas and the degree of fluorescein staining in each area was scored from 0 to 3 points according to the criteria described previously\(^\text{28}\) by an observer unaware of the treatment that the animals had received. The scores for all areas were summed to obtain the overall fluorescein score.

Tear volume was measured by cotton thread (ZONE-QUICK; Showa Yakuhin Kako Co., Ltd., Tokyo, Japan), according to the standard procedure used for clinical practice.\(^\text{29}\)

### Statistical Analysis

All values were expressed as the mean ± SEM or SD. One-way ANOVA followed by the Tukey test was used to evaluate differences between more than three groups. Differences were considered to be significant for values of \(P\) less than 0.05.

### Results

**Diclofenac-Dependent Protection Against Hyperosmolarity-Induced Cell Damage**

From a library of medicines already in clinical use, we screened for compounds able to suppress hyperosmolarity-induced cell damage in HCE cells; cells were treated with each drug under hyperosmotic conditions (150 mM NaCl, approximately 600 mOsm/L) and the viable cell number was determined by the MTT method. Among candidate compounds selected by this
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conditions rapidly activate NFAT5 to stimulate its nuclear localization.12 We next examined the effect of diclofenac on the nuclear localization of NFAT5 under our experimental conditions. As shown in Figure 4B, cells cultured under hyperosmotic conditions for 1 hour showed increased levels of NFAT5 in nuclear fractions but not in whole-cell fractions, suggesting that the nuclear localization of NFAT5 was altered by the hyperosmotic conditions. The results in Figure 4B also suggest that the treatment of cells with diclofenac (but not bromfenac) stimulates the nuclear localization of NFAT5 under hyperosmotic conditions but not under isotonic conditions.

To examine the activity of NFAT5 in cultured cells, we monitored the mRNA expression of bgt1. Figure 4C shows that the mRNA expression of bgt1 was upregulated in cells cultured under hyperosmotic conditions, as described previously.14 Furthermore, treatment of cells with diclofenac (but not bromfenac) for 6 hours upregulated the bgt1 mRNA expression under hyperosmotic conditions but not under isotonic conditions (Fig. 4C). Taken together, these results suggest that, under hyperosmotic conditions, the treatment of cells with diclofenac stimulates both the expression and nuclear localization of NFAT5, which in turn exerts a protective effect against the hyperosmolarity-induced cell damage via an increase in the activity of NFAT5.

It was reported that HCE cells treated with hyperosmolarity increase inflammatory cytokines (such as TNF-α) and MMPs (such as MMP-9)15,31 and we here examined the effect of diclofenac on hyperosmolarity-dependent upregulation of expression of TNF-α and MMP-9. As shown in Figures 4D and 4E, cells cultured under hyperosmotic conditions exhibited the upregulation of mRNA expression of INF-α and MMP-9 and simultaneous treatment of cells with diclofenac suppressed this induction.

Diclofenac-Dependent Amelioration of Corneal Surface Damage in a Rat Model of Dry Eye

On a rat model of dry eye produced by the removal of lacrimal glands, we examined if the topical administration of diclofenac (or bromfenac) to eyes reduced the extent of corneal surface damage. The three times daily topical administration of diclofenac (or bromfenac) to eyes was commenced 1 week after the removal of lacrimal glands, and corneal surface damage, assessed as a function of the intensity of fluorescein staining, was measured a further 4 weeks later. The volume of tear fluid was lower in rats subjected to lacrimal gland removal than sham-operated rats at both 1 and 5 weeks after the surgical intervention (Fig. 5A).

As shown in Figures 5B and 5C, the removal of lacrimal glands increased the level of fluorescein staining at the corneal surface, thus demonstrating that the intervention resulted in corneal surface damage. The topical administration of diclofenac (or bromfenac) clearly suppressed this increase in the level of staining (Figs. 5B, 5C). We also examined the level of corneal epithelial cell death by TUNEL assay. The topical administration of diclofenac (but not bromfenac) decreased the number of TUNEL-positive corneal epithelial cells in this rat model (Figs. 5D, 5E). It was confirmed that diclofenac (or bromfenac) did not further affect the volume of tear fluid in rats subjected to lacrimal gland removal (Fig. 5A). We also examined the effect of topical administration of diclofenac (or bromfenac) on expression of NFAT5. Immunohistochemical analysis with an antibody against NFAT5 showed that the level of NFAT5 at the corneal surface was increased by the topical administration of diclofenac (but not bromfenac) (Figs. 5F, 5G) in the rat dry eye model. In summary, these results in Figure 5 suggest that the topical administration of diclofenac to eyes suppresses the corneal surface damage produced by the removal of lacrimal glands, but without affecting the tear fluid volume.
DISCUSSION

Dry eye syndrome affects the quality of life of a large number of people around the world. As clinical protocols have not yet been established, there is scope for new types of drugs to be developed. Although it is clear that hyperosmolarity-induced ocular epithelial cell apoptosis plays an important role in the pathogenesis of DES, drugs that could suppress this apoptosis have not been identified at present. To address this issue, we tried to screen for such compounds.

FIGURE 1. Effects of different NSAIDs on hyperosmolarity-induced cell damage. Human corneal epithelial cells were incubated with the indicated concentration of each NSAID in the presence of 150 mM NaCl (A) or 280 mM glucose or sorbitol (B) for 24 hours. Viable cell number was determined by the MTT method and expressed relative to control (isotonic conditions) absorbance value. Values are mean ± SD, n = 3. *P < 0.05; **P < 0.01.
Diclofenac eye drops are widely used for the treatment of ocular inflammation, such as keratitis, conjunctivitis, uveitis, and postoperative inflammation. Compared with corticosteroids and cyclosporine A, topical administration of diclofenac to eyes has certain safety advantages, being free of the adverse effects associated with the use of corticosteroids (such as an increase in IOP, lens opacification, and cataractogenesis) or cyclosporine A (such as burning symptoms and conjunctival hyperemia). It was reported that the level of PGE2 in tear fluid was increased in DES patients compared with healthy volunteers, and that some NSAIDs (such as bromfenac sodium [bromfenac]) were found to be effective suppressors of inflammation associated with DES. These results suggest that the topical administration of diclofenac to eyes may be a good candidate therapy for DES; however, such idea had not been tested.

We here found that treatment with diclofenac protected cultured HCE cells against hyperosmolarity-induced cell damage via the suppression of both apoptosis and cell growth arrest. This protective effect was not, however, observed for the other NSAIDs tested and there was no evident relationship between protective effects and the capacity of NSAIDs to inhibit COX activity or of their COX-1/COX-2 selectivity (COX-2-selective NSAIDs are celecoxib, rofecoxib, and meloxicam; COX-1-selective NSAID is SC-560). These findings suggested that diclofenac suppresses hyperosmolarity-induced cell damage independently of COX inhibition accompanied by a decrease in the level of PGE2. The following evidence was obtained in support of this conclusion: (1) exogenously added PGE2 did not affect the viable cell number, even at concentrations much higher than the endogenous level, in the presence of diclofenac under hyperosmotic conditions; (2) the dose-response profiles of diclofenac describing its protective effect against hyperosmolarity-induced cell damage was not correlated with that of the decrease in the level of PGE2; and (3) although bromfenac decreased the PGE2 level in a similar way to that of diclofenac, it did not protect against hyperosmolarity-induced cell damage.

It was previously reported that cells respond to hyperosmotic stress via the NFAT5-mediated induction of expression of various genes (such as transporters of compatible small organic osmolytes); this induction is mediated by stimulation of both the expression and nuclear localization of NFAT5. We found here that treatment of cells with diclofenac for 1

![Figure 2](https://iov.s.org/doi/10.1126/iovs.13-13484-supplement1)
hour stimulated the nuclear localization of NFAT5 and that treatment for 6 hours stimulated the expression of NFAT5 under hyperosmotic conditions. We also found that treatment of cells with diclofenac upregulated the expression of bgt1 mRNA. We consider that the diclofenac-dependent stimulation of both the expression and nuclear localization of NFAT5 is involved in the activation of NFAT5 transcriptional activity.

**FIGURE 3.** Independence on COX inhibition of the protective effect of diclofenac against hyperosmolarity-induced cell damage. Human corneal epithelial cells were incubated with the indicated concentration of PGE2 (A), diclofenac (A, C), and bromfenac (C) in the presence of 150 mM NaCl for 24 hours. Viable cell number was determined by the MTT method and expressed relative to control (isotonic conditions) absorbance value. (B) Human corneal epithelial cells were preincubated with the indicated concentration of diclofenac or bromfenac for 30 minutes and further incubated with 10 μM arachidonic acid for 30 minutes in the presence of the same concentration of each NSAID as in the preincubation step. The amount of PGE2 in the culture medium was determined by EIA. Values are mean ± SD, n = 3. *P < 0.05; **P < 0.01.
nuclear localization, or increase bgt1 mRNA expression. These findings suggest that these phenomena play important roles in the protection shown by diclofenac against hyperosmolarity-induced cell damage. It should be noted that diclofenac affected neither the expression nor nuclear localization of NFAT5 under normal osmotic conditions. Thus, it seems that diclofenac specifically affects cells exposed to hyperosmotic stress, which is an interesting outcome considering its possible clinical application for the treatment of DES patients.

**Figure 4.** Effect of diclofenac on the expression and nuclear translocation of NFAT5. Human corneal epithelial cells were incubated with the indicated concentration of diclofenac (A-E) or bromfenac (A-C) in the presence or absence of 150 mM NaCl for 6 hours (A, C), 1 hour (B), 12 hours (D), or 24 hours (E). Whole-cell extracts (A, B) or nuclear extracts (B) were analyzed by immunoblotting with an antibody against NFAT5, actin, or lamin B (A, B). The intensity of the NFAT5 band was determined and expressed relative to the control (isotonic conditions without NSAID) value (A, B). The relative expression of bgt1 (C), tnf-a (D), or mmp-9 (E) mRNA was monitored by real-time RT-PCR. (C-E) Values normalized to actin expression were expressed relative to the control (isotonic conditions without NSAID) value. Values are mean ± SD, n = 3. *P < 0.05; **P < 0.01.
FIGURE 5. Effects of diclofenac eye drops on corneal surface damage in rats. The rat model of dry eye was developed by the removal of lacrimal glands, and the diclofenac or bromfenac treatment was administered from weeks 1 to 5 after removal. (A–G) Diclofenac (0.1%, 3.1 mM), bromfenac (0.1%, 2.6 mM), or vehicle (the same buffer without NSAID) eye drops (5 μL) were administered three times daily. (A) The volume of tear fluid was measured according to cotton thread test. (B) Images of fluorescein-stained cornea are shown. (C) Fluorescein score was calculated as described in materials and methods. (D–G) Sections of eye tissues were prepared 5 weeks after lacrimal gland removal. (D) Sections were subjected to TUNEL assay and DAPI staining (scale bars: 50 μm). (E) The number of TUNEL-positive cells was counted. (F) Immunohistochemical analysis with an antibody to NFAT5 was performed (scale bars: 50 μm). (G) The relative fluorescence intensity was determined. Values are mean ± SEM. *P < 0.05; **P < 0.01. n.s., not significant.
In the rat model for DES, the topical administration of diclofenac (0.1%) to eyes suppressed the corneal surface damage caused by removal of the lacrimal glands without affecting the tear fluid volume. Furthermore, administration of bromfenac by the same protocol did not reduce the damage. In agreement with our in vitro results, these results suggest that diclofenac suppressed corneal surface damage by protecting the corneal epithelial cells against hyperosmolarity-induced damage. As diclofenac eye drops (0.1%) have been used for the treatment of ocular inflammation, the safety of this treatment approach has thus already been confirmed in humans. This provides diclofenac with a distinct advantage for its clinical use to treat DES compared with new agents that have yet to be clinically tested or approved.

A concern for use of diclofenac eye drops is corneal melting. We reported that topical administration of diclofenac for 2 months was free from significant abnormality in corneal epithelial structure and function in patients with healthy corneas, but this topical administration caused significant abnormality in some patients with refractive surgery. However, as the pathogenesis of diclofenac (or other NSAID)-induced corneal melting is unclear at present, we could not predict the risk of diclofenac-dependent corneal melting in DES patients. Therefore, at the clinical study to test the efficacy of diclofenac eye drops on DES patients, corneal melting should be paid much attention.

As described in the introduction, inflammation plays an important role in the corneal surface damage associated with DES. Thus, the ineffectiveness of bromfenac eye drops to combat corneal surface damage induced by the removal of the lacrimal glands is surprising. We consider that this is because inflammation was not induced in this animal model of dry eye under the conditions used. In fact, we found that the mRNA expression of proinflammatory cytokines (such as TNF-α) was not induced in the eyes of rats subjected to lacrimal gland removal (data not shown). An absence of inflammatory responses was also reported in a monkey model of dry eye induced by lacrimal gland removal. On the other hand, the anti-inflammatory effect of NSAIDs could be effective in suppressing inflammation associated with DES in humans. For example, a phase 2 clinical study of bromfenac eye drops to treat DES noted the efficacy of this drug. Thus, the advantage of diclofenac eye drops to treat DES is that this drug not only has anti-inflammatory effects but also cytoprotective actions. On this basis, we propose that diclofenac eye drops could be therapeutically beneficial for DES patients.

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**References**


