Improvement of Corneal Barrier Function by the P2Y<sub>2</sub> Agonist INS365 in a Rat Dry Eye Model

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**PURPOSE.** Because purinoceptor P2Y<sub>2</sub> receptor agonists elicit increases in net Cl, fluid transport, and glycoprotein release onto the ocular surface, they are candidates for treatment of dry eye syndrome. Accordingly, the effects of such an agonist INS365 on these parameters were characterized in a rat dry eye model.

**METHODS.** An SD rat dry eye model was used in which exorbital lacrimal gland extirpation decreased the Schirmer test score by at least 50%. After 8 weeks, when significant increases occurred in corneal epithelial permeability, INS365-containing eye drops were applied six times daily for the next 4 weeks at concentrations from 0.03% to 3.0%. Corneal barrier function was evaluated based on measurements with a modified anterior fluorometer of fluorescein penetration at 1, 2, and 4 weeks after initial application. After INS365 application, the periodic acid–Schiff reagent (PAS)-stained area was evaluated in histologic sections of the tarsal and bulbar conjunctiva.

**RESULTS.** Ten minutes after INS365 eye drop application at doses of either 3.0% or 8.5%, a 1.5-fold transient increase in tear fluid secretion occurred in both the control and dry eye model animals. These transient increases nearly returned to baseline after 60 minutes. Furthermore, after 5 minutes, 1.0% INS365 was sufficient to cause a maximal transient decrease in the PAS-stained area of more than 30%, which thereafter recovered toward the initial level. Beginning at 2 weeks and continuing for an additional 2 weeks, maximal declines in dye penetrance of approximately 50% occurred with doses of INS365 as low as 1%. Such improvement in corneal epithelial resistance was accompanied by complete restoration of the PAS-stained area to the level seen in the control animal.

**CONCLUSIONS.** In a rat dry eye model, the P2Y<sub>2</sub> agonist INS365 was found to improve surface health, based on increases in tear fluid secretion, corneal epithelial resistance, and release of glycoprotein-containing moieties from goblet cells. These effects suggest that INS365 is a potential therapeutic agent for use in the treatment of dry eye syndrome. (*Invest Ophthalmol Vis Sci.* 2001;42:96–100)

**MATERIALS AND METHODS**

**Ophthalmic Solutions**

INS365 was supplied by Inspire Pharmaceuticals (Durham, NC). INS365-containing eye drops were formulated in distilled water at a concentration of 0.1% to 8.5% (wt/vol), and the osmolarity was adjusted with NaCl to 280 to 300 mOsm.

**Animals**

Male 6-week-old Sprague–Dawley rats (SLC Japan Shimizu, Shizuoka, Japan) were kept under standard pathogen-free conditions in constant periods of 12 hours light and 12 hours dark (23°C) and were allowed access to food and water ad libitum. All studies adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Tear Fluid Secretion Measurement**

Five microliters of vehicle (0.9% saline) or 0.1%, 0.3%, 1.0%, 3.0%, or 8.5% INS365-containing eye drops were applied to the rats’ conjunc-
tival sacs. Tear fluid secretion was measured with Schirmer strips (Showa Yakuhin Kako, Tokyo, Japan) 10 to 60 minutes after application of eye drops. A 1 × 17-mm strip was inserted into the lower eyelid, and the rat was released on the tray (60 × 60 cm) for 1 minute. Strip-wetting length was measured to an accuracy of 0.5 mm.

**Glycoprotein Detection in Goblet Cells**

Animals were killed and then fixative solution (2.5% formalin-3% glutaraldehyde solution [F-G] at a ratio of 1:15 moles phosphate buffer, pH7.2) was instilled into the conjunctiva to fix the ocular surface. The attached tarsal and bulbar conjunctiva were removed from the eyes and fixed with F-G fixative solution for 16 hours. Subsequently, these tissues were transferred into 10% buffered formalin for 24 hours. The conjunctiva and the globe were embedded in paraffin and 5 μm vertical sections through the papillary-optic nerve head plane were stained with periodic acid–Schiff reagent (PAS) using a standard protocol. The areas were averaged from three different sections of goblet cells in the upper and lower conjunctiva. In each sample, the microscopic images of bulbar-palpebral conjunctival epithelium were digitized. The PAS-stained areas were selected by color to extract the goblet cells and were evaluated with computer software (Win Roof; Mitani, Fukui, Japan).

**Corneal Epithelial Permeability Measurement in Rat Dry Eye Model**

To establish the dry eye model, the rats were intraperitoneally anesthetized with pentobarbital sodium (40 mg/kg) followed by surgical bilateral removal of the exorbital lacrimal glands. In these animals, the Schirmer score decreased to approximately half the value measured in untreated animals. After 2 months, they received eye drops six times daily containing either 5 μl of vehicle (0.9% saline) or 0.03%, 0.1%, 0.3%, 1.0%, or 3.0% INS365 for 4 weeks. Corneal barrier function was evaluated at 1, 2, and 4 weeks based on measurement of fluorescein penetrance. The fluorophotometric methods were modified for rats based on a previous report. Briefly, rats were anesthetized with pentobarbital sodium (35 mg/kg, intraperitoneally), and then 5 μl of a 0.5% fluorescein sodium solution was instilled into the conjunctival sac. The eyes were kept closed for 10 minutes, and then the excess fluorescein was washed out with saline. The eyes were held closed for an additional 20 minutes. Fluorescein penetration into the central cornea was measured with a slit lamp fluorophotometer (Anterior Fluorometer FL-500; Kowa, Nagoya, Japan), which was modified for rats. The instrument was focused on the central cornea, and the measurement angle was 60°. An area of 0.023 mm² was used, and the fluorescein intensity was measured five times and averaged. The fluorescein penetrance is expressed in terms of photon counts per millisecond.

**Statistical Analysis**

Data are presented as the mean ± SEM. Statistical comparisons were made either by Dunnett multiple comparison test after one-way analysis of variance (ANOVA) or by Student’s t-test. P < 0.05 was considered statistically significant.

**RESULTS**

**Effect of INS365 on Tear Secretion in Normal Subjects**

The results shown in Figure 1A indicate that in four normal rats, the Schirmer score maximally increased in eight eyes by 138% after treatment with either 3.0% or 8.5% INS365. The time dependence of this maximal effect with 8.5% INS365 is shown in Figure 1B and indicates that the increases were maximal at 10 minutes and then showed a gradual, slight decline after another 50 minutes. Application of the vehicle (0.9% saline) did not affect the Schirmer score.

**Effect of INS365 on Glycoprotein Release in Normal Rats**

Figure 2 compares typical conjunctival patterns of PAS staining 5 minutes after application of either INS365 or saline in the same group of four normal rats. In the untreated rat, the conjunctiva had a number of glycoprotein-containing goblet cells (Fig. 2A). In the saline-treated animal, there was a slight decline in glycoprotein staining (Fig. 2B). On the contrary, in the INS365-treated group more goblet cells lost glycoprotein staining (Fig. 2C). Figure 3A shows the time course of the changes in PAS-stained areas after a single application of either saline or 8.5% INS365-containing eye drops. The PAS-stained areas significantly decreased in the 8.5% INS365-treated group 5 minutes after application and fully returned to normal within another 25 minutes. Although 0.9% saline slightly decreased the PAS-stained area, it was not significant. Figure 3B shows the dose-dependent effects of INS365 (0.1–8.5%) on the PAS-stained area 5 minutes after application. INS365 at 1.0% maximally decreased the stained area. However, the sensitivity of the assay may not have been sufficient to detect a significant decline in stained area after this time.

**Effect of INS365 on Tear Secretion in a Dry Eye Model**

To determine whether INS365 could also increase tear secretion in our rat dry eye model, the Schirmer test was performed 10 minutes after eye drop application. As in normal animals, the results shown in Figure 4 indicate that 3.0% and 8.5% INS365 increased tear fluid secretion. The increase with 8.5% INS365 was slightly larger than that with the 3.0% solution and was identical with the increase obtained in the normal animal. However, at INS365 concentrations less than 3.0%, Schirmer test scores did not change.

**Restorative Effect of INS365 on Corneal Epithelial Permeability in Dry Eye Model**

The results shown in Figure 5 indicate that 2 months after surgery in the dry eye model, fluorescein penetration into the stroma was approximately 3.5-fold higher than that in the normal eye. To determine whether INS365 could mitigate this increase, the dose- and time-dependent effects were deter-
mined of INS365 on restoration of corneal epithelial permeability. Figure 5 compares the effects of INS365 (0.03–3.0%) at 1, 2, and 4 weeks after the first INS365 instillation. Corneal epithelial permeability restoration occurred in a dose-dependent fashion. Relative to the normal control, the vehicle-treated dry eye model had a sustained increase in fluorescein permeability throughout the entire period. On the contrary, corneal permeability significantly decreased as early as 1 week after treatment with 3.0% INS365 was initiated. With 0.1% to 1.0% INS365, the declines in corneal epithelial permeability relative to the vehicle-treated group all became significant after 2 weeks and remained stable for another 2 weeks. The effects of 1.0% and 3.0% INS365 were indistinguishable from one another. The largest restorative effect achieved with INS365 resulted in a decline in corneal epithelial permeability of 51% from the value measured in the treated control.

To determine in the dry eye model whether there is an association between an INS365 mediated decrease in corneal epithelial permeability and release of glycoprotein onto the tear surface, we examined the PAS-stained area after 4 weeks. This examination was performed because a decline in goblet cell density is a hallmark of dry eye disease. On the day after measuring fluorescein permeability, PAS-stained area in the 0.3% to 3.0% INS365-treated rats was evaluated, because it is an indicator of goblet cell density. The results shown in Figure 6 indicate that in the treated group, the PAS-stained area decreased by approximately 30% from the untreated control. The application of saline had no effect on the PAS-stained area. However, interestingly, in the INS365-treated groups (0.3%, 1.0%, and 3.0%) the PAS-stained area increased dose dependently. In the 1.0% INS365-treated group, the PAS-stained area recovered after 4 weeks to its control level, whereas saline treatment did not increase the PAS-stained area. These results suggest that in this dry eye model INS365 application can improve ocular surface health by fully restoring the normal ocular surface glycoprotein-containing content.

**DISCUSSION**

We report the use of a new rat dry eye model to evaluate the effectiveness of the P2Y2 receptor agonist INS365 to improve tear secretion and restore corneal epithelial barrier function. Our methods of evaluating dry eye severity are indirect rather than using fluorescein or rose bengal staining. Nevertheless, they are appropriate, because they can be used to evaluate tear secretion and corneal barrier function. The methodology of barrier function determination involves use of a modified slit lamp fluorophotometer to quantify fluorescein penetration. The method is simple, and its results can be correlated with the clinical grading of superficial punctate keratopathy in patients.
with dry eye. Therefore, our approach provides meaningful insight into whether a compound is a potential candidate for use in dry eye therapy. We found with this model that the repeated application of INS365-containing eye drops ameliorated some of the clinical signs associated with desiccation of the ocular surface. This improvement in ocular surface health occurred as the result of increases in tear fluid and restoration of apparent ocular surface cells with glycoprotein-containing moieties. Such effects are consistent with the declines in fluorescein penetrance, which is indicative of recovery of epithelial membrane permeability and tight junctional resistance. Given these effects, INS365 is a candidate for further development as a therapeutic agent in the treatment of dry eye.

INS365 increased tear secretion in normal rats and in our dry eye model (Figs. 1, 4), which is in agreement with its effect in normal rabbits. The maximal increases occurred after 10 minutes with same INS365 concentrations in each case: 138% and 170%, respectively. The transient stimulated level in the dry eye model was nearly the same as the baseline value in normal rats. Because the increases in Schirmer test scores were essentially the same in the normal and dry eye model rats, it appears that INS365 increases fluid secretion through the stimulation of net ion transport across the conjunctiva rather than the lacrimal gland. Such an effect of INS365 has been described on net Cl transport and water transport from the stroma to the tear-side bathing solution in the isolated conjunctiva. Nevertheless, it is also possible that INS365 has in addition a stimulatory effect on accessory lacrimal gland and/or meibomian gland function.

Mucin secretion is regulated by neurotransmitters in rat conjunctival goblet cells. Similarly, the P2Y2 agonists UTP and ATP stimulate mucin release from goblet cells in several other tissues. Furthermore, P2Y2 receptor stimulation in the isolated conjunctiva increases mucin secretion. We also have shown in the living rabbit that application of INS365 increases release of glycoprotein-containing moieties and mucin secretion from conjunctival goblet cells into the tear fluid. It is not possible to know whether this INS365-induced effect results from a direct or an indirect stimulation of goblet cell function and/or density. An indirect effect is tenable because of increases in ocular surface hydration, which promotes ocular surface tissue health. In the normal rat, glycoprotein secretion was also stimulated by INS365 (Fig. 3). This response peaked 5 minutes after INS365 application, which is the same time as that needed for UTP to elicit such an effect in rabbits. The subsequent decline within 30 minutes in PAS-stained area toward the control level suggests that there may be a rapid turnover of glycoprotein-containing moieties elaborated from conjunctival goblet cells onto the tear surface. This suggestion is in agreement with actual measurements of turnover in the intestine. The repeated administration of INS365 at intervals of more than 1 hour may be long enough to sustain tear glycoprotein-containing moiety content such as mucin at levels that are higher than those reported in the tears of normal animals. Such an effect promotes ocular surface hydration. In fact, gefarnate, which stimulates mucin secretion and synthesis, protects against ocular surface desiccation in the rabbit short-term dry eye model. Similarly, INS365 induced increases in tear and mucin secretion and protected the ocular surface from desiccation and losses of corneal epithelial resistance.

Dry eye syndrome is an ocular surface disorder associated with tear film abnormalities. For the treatment of patients with dry eye syndrome, ocular surface hydration can be supported through the repeated application of artificial tear formulations and the wearing of moisture-retaining glasses. However, these therapies provide only partial relief. Another approach of potential therapeutic benefit could be to use INS365-containing eye drops, which may provide additional relief through the stimulation of tear fluid and mucin secretion.

Acknowledgment

The authors thank Peter S. Reinach for technical assistance and critical review of the manuscript.

References