RESTORATION OF CORNEAL EPITHELIAL BARRIER FUNCTION AND WOUND HEALING BY SUBSTANCE P AND IGF-1 IN RATS WITH CAPSAICIN-INDUCED NEUROTROPHIC KERATOPATHY

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PURPOSE. To investigate the effects of topical application of the combination of substance P (SP) and insulin-like growth factor (IGF)-1 on corneal epithelial barrier function and epithelial wound closure in rats with capsaicin-induced neurotrophic keratopathy.

METHODS. Neonatal rats were injected subcutaneously with a single dose of capsaicin to induce neurotrophic keratopathy. Corneal epithelial barrier function was evaluated with an anterior fluorophotometer. Tear fluid secretion was measured by the Schirmer test. Corneal epithelial wound healing was determined by measurement of the size of the epithelial defect after debridement of the entire epithelium. The combination of SP (1 mM) and IGF-1 (1 µg/mL) in phosphate-buffered saline was administered in eye drops six times daily.

RESULTS. Corneal epithelial barrier function was impaired and corneal epithelial wound healing was delayed in rats injected with capsaicin. The application of eye drops containing the combination of SP and IGF-1 to capsaicin-injected rats resulted in a significant improvement in corneal epithelial barrier function compared with that apparent in capsaicin-injected animals that received eye drops containing vehicle alone. Such treatment with SP and IGF-1 also significantly increased the rate of corneal epithelial wound closure in capsaicin-injected animals.

CONCLUSIONS. Topical application of the combination of SP and IGF-1 improved both corneal epithelial barrier function and epithelial wound healing in an animal model of neurotrophic keratopathy. (Invest Ophthalmol Vis Sci. 2003;44:2937–2940) DOI:10.1167/iovs.02-0868

Corneal innervation is important for the maintenance of corneal structure and function. Corneal nerve damage in animals thus results in physiological abnormalities of the corneal epithelium, such as an increase in permeability, a decrease in cell proliferation, changes in cellular phenotype, and delayed wound healing.1-3 Various types of corneal injury or disease in humans, including trauma, herpetic keratitis, and diabetic keratopathy, result in the development of neurotrophic keratopathy or keratitis, which is often accompanied by persistent corneal epithelial defects or trophic ulcer.4-6 These observations suggest that factors released from sensory nerves may play an important role in the physiology of the corneal epithelium.

We have shown that substance P (SP) and insulin-like growth factor (IGF)-1 synergistically promote migration of the rabbit corneal epithelium in an organ culture system.7 Although neither agent alone affected corneal epithelial migration, their synergistic effect was dose dependent. The combination of SP and IGF-1 also synergistically promotes corneal epithelial wound closure in vivo in normal rabbits.8 On the basis of these laboratory findings, we have successfully treated persistent epithelial defects in individuals with Riley-Day syndrome9 or neurotrophic keratopathy10,11 by the application of eye drops containing both SP and IGF-1.

The promotion of corneal epithelial wound closure in rabbits by the combination of SP and IGF-1 was demonstrated in animals with intact corneal innervation. It was not determined whether these agents facilitate epithelial wound closure in the denervated cornea. Animals exposed to capsaicin exhibit various morphologic changes in the cornea, including corneal lesions, a reduction in the number of corneal nerve fibers, and disintegration of epithelial cells.12-14 In addition, physiological changes, such as a reduction in tear fluid secretion, impairment of corneal epithelial barrier function, and a delay in corneal epithelial wound healing, are also apparent in such animals.15,16 These capsaicin-induced pathophysiological changes thus appear similar to those characteristic of humans with neurotrophic keratopathy.

We have now investigated the potential effects of the combination of SP and IGF-1 on the physiological and pathologic properties of the corneal epithelium in animals with compromised corneal innervation as a result of capsaicin administration. We measured changes in epithelial barrier function, tear fluid secretion, and epithelial wound closure in rats injected with capsaicin soon after birth and subsequently treated with eye drops containing SP and IGF-1 or those containing vehicle.

METHODS

Injection of Rats with Capsaicin

Pregnant Wistar/ST rats were obtained from Japan SLC (Hamamatsu, Shizuoka, Japan). Care and treatment of animals conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The general handling of animals and injection of neonatal rats with capsaicin were performed as previously described,12,15 with some modifications. On postnatal day 4, rats of both sexes were given a single subcutaneous injection of capsaicin (50 mg/kg of body mass; Sigma-Aldrich, St. Louis, MO) dissolved in physiological saline containing 10% ethanol and 10% Tween 80. Control rats received the vehicle solution alone. The injected rats were housed with their mothers, who were fed standard rat chow and maintained under normal conditions. At 5 to 6 weeks of age, the injected rats were separated according to sex, housed in cages containing three to five animals each, and maintained under normal conditions until the initiation of experiments. These animals exhibited mild superficial punctate keratopathy with sparse lesions and no neovascularization, consistent with the condition of similarly treated rats described previously.15 No changes were ap-
parent in the general behavior or body weight of rats injected with capsaicin, despite the existence of other SP-containing neurons in the body.

**Evaluation of Corneal Epithelial Barrier Function**

Two months after capsaicin injection, rats were divided into two groups (five animals per group). The vehicle group received eye drops containing phosphate-buffered saline (PBS) alone, and the experimental group was treated with eye drops containing 1 mM SP (Sigma-Aldrich) and IGF-1 (1 µg/ml; BD Biosciences, Bedford, MA) in PBS. In general, concentrations 10- to 100-fold greater than those effective in vivo are needed to evaluate the effects of growth factors and peptides in vivo. The concentrations of SP and IGF-1 used in the present study were thus 50 and 100 times, respectively, those shown to be effective in our previous in vitro experiments. We also previously confirmed the effectiveness of these doses in promoting wound closure in vivo. Both eyes received 5 µL of solution six times a day for 2 weeks. Corneal epithelial barrier function was evaluated by measurement of fluorescein penetrance, for 3 days and 1 and 2 weeks after the onset of installation of eye drops. The fluorophotometric method was based on that of a previous study but was modified for rats. It is simple and practical and it provides a quantitative measure of corneal epithelial damage, especially of that associated with superficial punctate keratopathy. Furthermore, the obtained values correlate well with the grading of corneal epithelial damage by slit lamp observation. In brief, rats were anesthetized with an intraocular injection of pentobarbital sodium (35 mg/kg), and 5 µL of 0.5% fluorescein sodium was then instilled into the conjunctival sac. The eyes were maintained closed for 10 minutes, after which the excess fluorescein was washed out with saline, and the eyes were kept closed for an additional 20 minutes. Fluorescein penetrance into the central cornea was then measured with a slit lamp fluorophotometer (Anterior Fluorometer FL-500; Kowa, Nagoya, Japan) that had been modified for rats. The instrument was focused on the central cornea, with a measurement angle of 60°. Fluorescence intensity was measured five times in a 0.023-mm² area and then averaged, and fluorescein penetrance was expressed in photon counts per millisecond. Preliminary time course experiments revealed that fluorescein penetrance was substantial and stable 8 weeks after injection of rats with capsaicin, which is why we initiated treatment with SP and IGF-1 2 months after capsaicin injection.

**Measurement of Tear Fluid Secrecion**

Tear fluid secretion was measured without topical anesthesia by a modified version of the Schirmer test in the animals subjected to assessment of corneal barrier function, both before and after the 2-week treatment period. The Schirmer strips (Showa Yakuhin Kako, Tokyo, Japan), cut to dimensions of 17 × 1 mm, were inserted below the lower eyelid of the test animal, which was then freed on a tray (60 × 60 cm) for 1 minute. The wet length of the strip was measured to an accuracy of ±0.5 mm.

**Evaluation of Corneal Epithelial Wound Healing**

Two months after injection of capsaicin, rats were anesthetized by an intraperitoneal injection of pentobarbital sodium and the application of topical oxybuprocaine drops to each eye. The corneal epithelium was scraped off from limbus to limbus with a dull scalpel blade. The vehicle group (five rats) received eye drops consisting of PBS alone, and the experimental group (five rats) was treated with eye drops containing 1 mM SP and IGF-1 (1 µg/ml) in PBS. Both eyes received 5 µL of solution six times a day for 4 days, beginning immediately after epithelial debridement. The epithelial defect was stained with one drop of 1% fluorescein and photographed every 12 hours after debride ment, and its area was measured on the photographs with a computer-assisted image analyzer. The healing rate of each eye was calculated by linear regression analysis of the data collected between 12 and 36 hours after epithelial debridement. One of the corneas of a rat in the vehicle group was perforated during epithelial scraping, and this animal was therefore excluded from further evaluation.

**Statistical Analysis**

Experiments were performed in a double-blind manner to avoid potential bias. Data are expressed as means ± SEM of results from 8 to 10 determinations. Statistical analysis was performed with the unpaired Student’s t-test for comparisons between two groups and with the Dunnett multiple comparison test for those among three groups. P < 0.05 was considered statistically significant.

**Results**

We first investigated the effect of the combination of SP and IGF-1 on corneal epithelial barrier function in rats injected with capsaicin. Fluorescein penetrance into the corneal stroma of rats injected 2 months previously with capsaicin (343 ± 33 and 352 ± 43 photon counts/ms for the vehicle and experimental groups, respectively) was approximately 2.6-fold greater than that apparent in control rats (129 ± 12 photon counts/ms; Fig. 1), indicative of an impairment of corneal epithelial barrier function in this experimental model of neurotrophic keratopathy. Treatment of capsaicin-injected rats for 3 days with eye drops containing SP and IGF-1 resulted in a marked decrease in fluorescein penetrance into the stroma. Continued treatment with SP and IGF-1 induced a further gradual decrease in fluorescein penetrance, although this parameter had not returned to baseline at the end of the 2-week treatment period. Fluorescein penetrance in capsaicin-injected rats that received eye drops containing vehicle alone as well as that in the control rats not injected with capsaicin remained largely unchanged during the observation period. The difference in fluorescein penetrance between capsaicin-injected rats treated with SP and IGF-1 (176 ± 55 photon counts/ms) and those that received vehicle (268 ± 24 photon counts/ms) was thus statistically significant 2 weeks after the onset of treatment. The impairment of corneal epithelial barrier function induced by injection of capsaicin was thus partially corrected by the application of eye drops containing both SP and IGF-1. We also examined the effect of SP and IGF-1 on tear fluid secretion in capsaicin-injected rats with the Schirmer test. Tear...
fluid secretion in rats injected 2 months previously with capsaicin (5.38 ± 0.59 and 5.50 ± 0.46 mm/min for the vehicle and experimental groups, respectively) was significantly decreased compared with that apparent in control rats (9.95 ± 0.60 mm/min; Fig. 2). Treatment of the capsaicin-injected rats for 2 weeks with eye drops containing both SP and IGF-1 had no effect on tear fluid secretion, which remained significantly decreased.

We next examined the effect of SP and IGF-1 on corneal epithelial wound closure in this model of neurotrophic keratopathy. Immediately after epithelial debridement, the size of the wound area in all animals was 20 to 22 mm². Epithelial wound healing was characterized by a lag phase comprising the first 12 hours after wounding, a relatively rapid rate of wound closure from 12 to 48 hours, and a final slower phase of repair from 48 to 96 hours in both capsaicin-injected and control rats (Fig. 3). However, 12 hours after epithelial debridement, the wound area of capsaicin-injected rats that received eye drops containing vehicle was significantly greater than that of animals not injected with capsaicin. This difference remained statistically significant at all time points up to 72 hours and was indicative of delayed epithelial wound healing in this model of neurotrophic keratopathy. In contrast, treatment of capsaicin-injected rats with eye drops containing SP and IGF-1 increased the rate of epithelial wound healing; the size of the wound area in such animals was thus significantly smaller between 12 and 60 hours after epithelial debridement than that in capsaicin-injected rats receiving vehicle eye drops. The healing process in the capsaicin-injected rats treated with the combination of SP and IGF-1 was similar to that in the control animals not injected with capsaicin. However, complete wound closure in rats treated with SP and IGF-1 had not been achieved by 96 hours after the onset of treatment. Calculation of the mean healing rates revealed that healing was significantly slower in the capsaicin-injected rats receiving vehicle eye drops than in control rats not injected with capsaicin (1.61% ± 0.119% vs. 2.04% ± 0.065% of wound area per hour; P < 0.01, Dunnett multiple comparison test). Moreover, the healing rate in capsaicin-injected rats treated with SP and IGF-1 (2.16% ± 0.169% of wound area per hour) was significantly greater than that in capsaicin-injected rats that received vehicle eye drops (P < 0.05, Student’s t-test). These results thus demonstrated that the delay in corneal epithelial wound healing in capsaicin-injected rats was reversed by treatment with the combination of SP and IGF-1.

**DISCUSSION**

Our data demonstrate that injection of rats with capsaicin results in an impairment of corneal epithelial barrier function, a reduction in tear fluid secretion, and a delay in corneal epithelial wound healing. Treatment of the injected animals with the combination of SP and IGF-1 restored corneal epithelial barrier function and normalized the rate of wound healing. The stimulatory effect of SP and IGF-1 on corneal epithelial wound healing in this animal model of neurotrophic keratopathy is consistent with that previously observed in normal rabbits.8

The administration of capsaicin to neonatal rats has previously been shown to induce morphologic and physiological changes in the corneal epithelium similar to those associated with neurotrophic keratopathy.12–15 Capsaicin induces desensitization of primary sensory neurons, resulting in the release and depletion of SP,17 which acts as a neurotransmitter or neuromodulator. The SP content and density of SP-containing nerve fibers in the cornea are both reduced markedly by administration of capsaicin in mice.18 Animals exposed to capsaicin thus provide a model for studies of the physiological roles of corneal innervation as well as of the pathophysiology of neurotrophic keratopathy. The dose of capsaicin (50 mg/kg) used in the present study appears appropriate for evaluation of the effects of test compounds on barrier function, tear secretion, and wound closure, given that the injected rats developed mild superficial punctate keratopathy with sparse lesions (grade 2 for corneal damage,19 approximately 300 photon counts/ms) and no neovascularization. We found that capsaicin doses of more than 75 mg/kg resulted in a more severe condition characterized by ulceration and neovascularization of the cornea (data not shown).

Eye drops containing both SP and IGF-1 were effective in treating the impairment of corneal epithelial barrier function and the delay of corneal epithelial wound healing in rats injected with capsaicin. However, although the combination of SP and IGF-1 significantly improved epithelial barrier function, the latter had not returned to baseline after treatment for 2
weeks, suggesting that complete restoration of epithelial barrier function may involve other factors. Furthermore, although the combination of these agents significantly increased the rate of corneal epithelial wound healing, it did not induce complete wound closure within 96 hours after epithelial debridement. Given that rapid resurfacing of epithelial defects is important for the relief of symptoms and for maintenance of normal corneal structure and function, an increase in the rate of wound closure is thought to be clinically beneficial in individuals with corneal disease. The combination of SP and IGF-1 did not affect the decrease in tear fluid secretion apparent in this animal model of neurotrophic keratopathy. Although diminished tear fluid secretion is thought to reflect a decrease in the afferent input responsible for the reflex that increases such secretion in response to corneal injury, its etiology remains undefined. Thus, although SP and IGF-1 improve corneal epithelial barrier function and epithelial wound healing, they may not increase corneal sensitivity in this animal model.

We have shown that SP and IGF-1 synergistically promote corneal epithelial migration in an organ culture system. This action of SP was not mimicked by other tachykinins or neuropeptides, and was shown to be mediated by NK-1 receptors for SP on corneal epithelial cells. The intracellular signaling responsible for the synergism of SP and IGF-1 appears to involve activation of the integrin, focal adhesion kinase, and paxillin system.

Neurotrophic keratopathy is characterized by various types of corneal epithelial disorder induced by trigeminal nerve palsy. Loss of corneal sensation thus results in corneal epithelial disorders such as superficial punctate keratopathy, erosion, and persistent epithelial defects. Although neurotrophic keratopathy is treated with artificial tears, tarsorrhaphy, or bandage soft contact lenses, none of these approaches is completely effective. Active medications for the treatment of this condition are thus needed.

A reduction in the SP content of the cornea has been shown to correlate with denervation of the trigeminal nerve to the eye. Our present results suggest that such a reduction in SP content resulting from corneal denervation may contribute to the development of neurotrophic keratopathy. Our data further indicate that the application of eye drops containing SP and IGF-1 may be an effective treatment for humans with neurotrophic keratopathy. Indeed, in a series of case reports, we previously showed that the topical application of SP or an SP-related peptide together with IGF-1 successfully treated individuals with neurotrophic or anhidrotic keratopathy. Topical treatment with nerve growth factor was also recently shown to restore corneal integrity in patients with corneal neurotrophic ulcers or keratitis. Further investigations of the possible clinical treatment of neurotrophic keratopathy with neurotrophic factors or with the combination of SP and IGF-1 are thus warranted.

References