Effects of Substance P and IGF-1 in Corneal Epithelial Barrier Function and Wound Healing in a Rat Model of Neurotrophic Keratopathy

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PURPOSE. To establish a rat model of neurotrophic keratopathy and to examine the effects of the combination of substance P (SP) and insulin-like growth factor (IGF)-1 on corneal epithelial barrier function and wound healing in this model.

METHODS. Corneal denervation was achieved by thermocoagulation of the ophthalmic branch of the trigeminal nerve. A modified Schirmer test was performed without topical anesthesia. Corneal epithelial barrier function was assessed by measurement of fluorescein permeability with an anterior fluorophotometer. Epithelial wound healing was evaluated by measurement of the area of the defect at various times after removal of the entire epithelium. Eye drops containing both 1 mM SP and IGF-1 (1 μg/mL) were administered six times daily.

RESULTS. The Schirmer test result in eyes subjected to trigeminal denervation was lower than that in control eyes. The fluorescein permeability of the corneal epithelium of denervated eyes was increased relative to that of control eyes. Furthermore, trigeminal denervation induced a delay in corneal epithelial wound healing. Application of eye drops containing SP and IGF-1 to denervated corneas restored the fluorescein permeability of the corneal epithelium to control levels and abolished the delay in epithelial wound healing.

CONCLUSIONS. A rat model of neurotrophic keratopathy, characterized by reduced tear secretion, loss of corneal sensation, impaired epithelial barrier function, and delayed epithelial wound healing, was established by trigeminal denervation. Treatment with both SP and IGF-1 improved corneal epithelial barrier function and stimulated corneal epithelial wound healing in this model. (Invest Ophthalmol Vis Sci. 2003;44:3810–3815) DOI:10.1167/iovs.03-0189

The cornea is the most heavily innervated tissue in the body. Corneal innervation is thought to be important for maintenance of a healthy corneal epithelium. Loss of corneal sensation as a result of damage to the trigeminal nerve often leads to neurotrophic keratopathy, which is associated with various types of corneal disorders, including superficial punctate keratitis, persistent epithelial defects, and stromal melting. Prompt and appropriate treatment for such disorders is important in individuals with neurotrophic keratopathy, to prevent serious complications such as corneal ulceration, perforation, and infection. However, no specific standard treatment regimen for neurotrophic keratopathy based on the pathobiology of this condition has been established. Rather, attempts have been made to manage the epithelial disorders with ocular lubricants, soft contact lenses, or tarsorrhaphy. Although corneal denervation due to trigeminal nerve injury has been shown to result in abnormalities in the physiology of the corneal epithelium, including increased permeability,1 decreased cell proliferation,2 phenotypic changes,1–3,4 and delayed wound healing1,4 in animals, no animal model has been available with which to examine the effects of drugs on neurotrophic keratopathy.

The healing of corneal epithelial wounds is modulated by various humoral factors and extracellular matrix proteins.5 Growth factors, such as epidermal growth factor6–10 and basic fibroblast growth factor,11,12 as well as interleukin-6,13,14 stimulate corneal epithelial migration both in vitro and in vivo. Components of the extracellular matrix, including fibronectin,15–19 hyaluronan,18,20 laminin,16 and collagen type IV,16,21 also facilitate epithelial migration. We have shown that substance P (SP) and insulin-like growth factor (IGF)-1 synergistically promote corneal epithelial migration in vitro22 and corneal wound closure in vivo.23 The combination of SP and IGF-1 also upregulates the expression of fibronectin receptors and induces the activation of protein kinases in corneal epithelial cells,24 and these actions are thought to contribute to the stimulation of corneal epithelial wound healing. However, the effect of SP and IGF-1 on corneal epithelial wound healing in animals with neurotrophic keratopathy has not been examined.

The purpose of the present study was therefore to establish an animal model of neurotrophic keratopathy and then to use this model to examine the effects of eye drops containing both SP and IGF-1 on the barrier properties of and wound healing in the corneal epithelium. For development of the animal model, we modified a technique described by Sigelman and Friedenwald2 to achieve trigeminal denervation in the rat.

METHODS

Animals
Male Brown Norway rats with body masses of 230 to 345 g were obtained from Japan Charles River (Yokohama, Japan). The study was performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Trigeminal Denervation
A schematic representation of the thermocoagulation track for trigeminal denervation of the right eye is shown in Figure 1. The left eye served as a control. Rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (35 mg/kg body weight) and then...
immobilized in a stereotaxic frame. The skull was exposed to reveal the sagittal suture and an opening was bored at a position 1.5 mm in front and 2 mm to the right of the bregma. The coagulating electrode, which was fabricated from an insulated needle with 2 mm of the tip exposed, was inserted vertically through the hole. The distance from the top of the skull to the ophthalmic branch of the trigeminal nerve is approximately 11 mm, and entry of the needle into the ophthalmic branch was indicated by the animal’s blinking. On the basis of the results of our preliminary studies, coagulation of the nerve was performed with a tip temperature (measured in saline with a thermometer) of 60°C for 105 seconds. After thermocoagulation, the hole was closed and the skin sutured, and penicillin potassium was injected intramuscularly. The blinking reflex of the rats was subsequently tested both before and 3, 7, 14, 21, and 28 days after trigeminal denervation, as described. In brief, rats were anesthetized by intraperitoneal injection of pentobarbital sodium (35 mg/kg), and 5 μL of 0.5% fluorescein was instilled into each eye. After 10 minutes, the eyes were washed with 10 mL of saline, and, after an additional 20 minutes, the intensity of fluorescein fluorescence in the corneal epithelium was measured. Fluorescein permeability was expressed as photon counts per millisecond. When the intensity exceeded the measurable limit, 1000 photon counts/ms was assigned.

**Figure 1.** Schematic representation of the thermocoagulation track for diathermy-induced trigeminal denervation of the right eye in the rat.

**Corneal Epithelial Barrier Function**

Corneal epithelial barrier function was evaluated by measurement of the intensity of fluorescein fluorescence in the corneal epithelium with an anterior fluorophotometer (FL-500; Kowa, Tokyo, Japan), both before and 3, 7, 14, 21, and 28 days after trigeminal denervation, as described. In brief, rats were anesthetized by intraperitoneal injection of pentobarbital sodium (35 mg/kg), and 5 μL of 0.5% fluorescein was instilled into each eye. After 10 minutes, the eyes were washed with 10 mL of saline, and, after an additional 20 minutes, the intensity of fluorescein fluorescence in the corneal epithelium was measured. Fluorescein permeability was expressed as photon counts per millisecond. When the intensity exceeded the measurable limit, 1000 photon counts/ms was assigned.

**Corneal Epithelial Wound Healing**

Two weeks after trigeminal denervation, rats were anesthetized by intraperitoneal injection of pentobarbital sodium (35 mg/kg) and the application of 0.4% oxybuprocaine hydrochloride to each eye. The entire epithelium of the cornea was then removed with a blunt blade. The epithelial defect was stained with 1% fluorescein and photographed every 12 hours after debridement. The area of the epithelial defect was measured on the photographs with a computer-assisted image analyzer. Changes in the area were plotted graphically, and the healing rates of denervated and control corneas were calculated by linear regression from the data obtained between 12 and 48 hours after debridement.

**Histopathology**

Rats were killed with an overdose of pentobarbital sodium 14 days after trigeminal denervation. All trigeminal nerves, subjected or not to thermocoagulation, were removed, fixed in formalin, and embedded in paraffin, and serial transverse sections were prepared and stained with hematoxylin-eosin and Klüver-Barrera solution. The sections were examined with a light microscope and photographed.

**Treatment with SP and IGF-1**

To examine the effect of the combination of SP and IGF-1 on corneal epithelial barrier function in eyes with trigeminal denervation, we administered eye drops (5 μL) comprising either vehicle (phosphate-buffered saline) or both 1 mM SP (Sigma, St. Louis, MO) and human recombinant IGF-1 (1 μg/ml; BD Biosciences, Bedford, MA) six times a day for 2 weeks beginning 2 weeks after denervation. To examine the effect of SP and IGF-1 on corneal epithelial wound healing, we applied the combination of these agents six times a day for 4 days, beginning immediately after debridement of the corneal epithelium (which was performed 2 weeks after denervation).

**Statistical Analysis**

Data are expressed as means ± SEM and were analyzed by Student’s t-test or two-way analysis of variance for repeated measures. P < 0.05 was considered statistically significant.

**RESULTS**

**Histopathologic Observations**

Photomicrographs of sections prepared from trigeminal nerves subjected to thermocoagulation revealed the lesions to be circumscribed in the medial portion of the nerve (Fig. 2A). The axons of the neurons located in the region of nerve damage had either disappeared or were swollen (Fig. 2C). In contrast, in the trigeminal nerves not subjected to thermocoagulation, the axons were readily apparent, and the neurons did not show eylid near the medial canthus for 1 minute. The wet length of the strip was then measured.

**Schirmer Test**

A modified Schirmer test was performed without topical anesthesia, both before and 3, 7, 14, 21, and 28 days after trigeminal denervation. The Schirmer strips, cut to a length of 17 mm and a width of 1 mm (Showa Yakuhin Kako, Tokyo, Japan), were inserted into the lower
any signs of damage (Figs. 2B, 2D). Staining of the myelin sheath with Kluver-Barrera solution revealed demyelination and proliferation of Schwann cells in the trigeminal nerves subjected to thermocoagulation (Fig. 2E), whereas those of control eyes exhibited a normal staining pattern (Fig. 2F).

Effect of Trigeminal Denervation on Schirmer Test Result

A modified Schirmer test without topical anesthesia was performed in rats both before and at various times after trigeminal denervation. The Schirmer test result in control eyes remained constant during the period of analysis (Fig. 3). In contrast, the Schirmer test result in eyes subjected to trigeminal denervation was significantly reduced compared with that in control eyes 3, 7, 21, and 28 days after denervation.

Effect of Trigeminal Denervation on Corneal Epithelial Barrier Function

Four weeks after denervation, slit lamp examination with fluorescein staining revealed an irregular epithelial surface over a large portion of the cornea of eyes subjected to trigeminal denervation (Figs. 4A, 4B). Corneal epithelial barrier function was also assessed by measurement of the intensity of fluorescein fluorescence in the corneal epithelium with an anterior fluorophotometer. Damage to the epithelial barrier resulted in an increase in fluorescein permeability. The fluorescein permeability of the corneal epithelium of eyes with trigeminal denervation was significantly increased compared with that of control eyes 3, 7, 14, and 28 days after denervation (Fig. 4C).

FIGURE 2. Histopathologic analysis of transverse sections of trigeminal nerves subjected to thermocoagulation. Trigeminal nerves, subjected (A, C, E) or not (B, D, F) to thermocoagulation, were isolated 14 days after the procedure, fixed, and embedded in paraffin, and serial transverse sections were stained with hematoxylin-eosin (A–D) or Kluver-Barrera solution (E, F). Nerves subjected to thermocoagulation exhibited loss, swelling, or clumping of axons as well as demyelination (arrows). Scale bars, 100 μm.

FIGURE 3. Effect of trigeminal denervation on Schirmer test result. Rats were subjected to a modified Schirmer test without topical anesthesia, both before and at the indicated times after trigeminal denervation of the right eye. The Schirmer test result was determined for denervated (ﬁlled bars) and control (open bars) eyes. Data are expressed as the wet length of the Schirmer strip measured after 1 minute and are the mean ± SEM of results in 12 eyes. *P < 0.05, **P < 0.01 versus the corresponding value in control eyes.

FIGURE 4. Effect of trigeminal denervation on fluorescein permeability of the corneal epithelium. Slit lamp examination with fluorescein staining was performed after 4 weeks in the eye of a rat subjected to trigeminal denervation (B) and the corresponding control eye (A). (C) Corneal epithelial barrier function of denervated (ﬁlled bars) and control (open bars) eyes was assessed by measurement of fluorescein fluorescence with an anterior fluorophotometer, both before and at the indicated times after denervation. Fluorescein permeability of the corneal epithelium is expressed as photon counts per millisecond, and data are the mean ± SEM of results in 12 eyes. *P < 0.05, **P < 0.01 versus the corresponding value in control eyes.
Trigeminal denervation thus impaired corneal epithelial barrier function.

**Effect of Trigeminal Denervation on Corneal Epithelial Wound Healing**

The entire corneal epithelium was removed from control and denervated eyes 2 weeks after trigeminal denervation. Epithelial wounds of control eyes were completely resurfaced 60 hours after debridement (Fig. 5). In contrast, resurfacing of the defects of denervated eyes was not complete, even 96 hours after epithelial debridement. There was a significant interaction between group (control or denervated eyes) and time after debridement by two-way analysis of variance for repeated measures (P < 0.01). The wound area of denervated eyes was significantly larger than that of control eyes at 24, 48, 60, 72, 84, and 96 hours after epithelial debridement. The healing rate measured between 12 and 48 hours after debridement in denervated eyes was significantly reduced compared with that in control eyes (0.417 ± 0.037 vs. 0.495 ± 0.019 mm²/h). The height value for denervated eyes treated with vehicle.

**Effects of SP and IGF-1 on Corneal Epithelial Barrier Function and Wound Healing in Denervated Eyes**

Finally, we evaluated the effects of eye drops containing both 1 mM SP and IGF-1 (1 μg/mL) on corneal epithelial barrier function and wound healing in eyes subjected to trigeminal denervation. The fluorescein permeability of the corneal epithelium in denervated eyes treated with SP and IGF-1 for 2 weeks was significantly less than that in denervated eyes treated with vehicle and was similar to that in nondenervated control eyes (Fig. 6). The application of SP and IGF-1 thus improved corneal epithelial barrier function.

Similarly, the administration of SP and IGF-1 to denervated eyes stimulated the resurfacing of corneal epithelial wounds compared with healing in denervated eyes treated with vehicle (Fig. 7). There was a significant interaction between group (vehicle or treatment with SP and IGF-1) and time after debridement by two-way analysis of variance for repeated measures (P < 0.01). The size of the epithelial defect in denervated eyes treated with SP and IGF-1 was significantly smaller than that in denervated eyes treated with vehicle at 24, 36, and 48 hours after debridement. The healing rate measured between 12 and 48 hours after epithelial debridement was significantly greater (P < 0.01) in denervated eyes treated with SP and IGF-1 (0.472 ± 0.012 mm²/h) than in those treated with vehicle (0.379 ± 0.023 mm²/h). The healing rate in the latter eyes was significantly reduced (P < 0.01) compared with that apparent in nondenervated eyes (0.487 ± 0.020 mm²/h; n = 9).

**DISCUSSION**

Our results have demonstrated that trigeminal denervation impairs the production of tear fluid, corneal epithelial barrier function.
function, and corneal epithelial wound healing in rats. Animals subjected to trigeminal denervation thus exhibited characteristics similar to those of humans with neurotrophic keratopathy. The administration of eye drops containing both SP and IGF-1 improved corneal epithelial barrier function and promoted corneal epithelial wound healing in this animal model of neurotrophic keratopathy.

The trigeminal nerve is thought to exert a trophic effect on the corneal epithelium. Denervation of the cornea has thus been shown to result in a 20% decrease in the number of mitotic epithelial cells as well as in a reduction in the intracellular abundance of acetylcholine. Such effects have been proposed to underlie the development of neurotrophic keratopathy. SP is a neuropeptide that is present in the cornea and is depleted by corneal sensory denervation. It also stimulates corneal epithelial cell growth. We have shown that SP and IGF-1 synergistically facilitate corneal epithelial migration but do not affect the uptake of [3H]thymidine by corneal epithelial cells. The combination of SP and IGF-1 also upregulates the expression of the integrin-α5 and -β1 chains, which together form the fibronectin receptor and induce the phosphorylation of focal adhesion kinase and paxillin in human corneal epithelial cells. These latter effects promote the attachment of corneal epithelial cells to the extracellular matrix. The combination of SP and IGF-1 is thus thought to facilitate corneal epithelial wound healing by stimulating the adhesion and migration of epithelial cells. In contrast, neither acetylcholine, vasoactive intestinal peptide, nor calcitonin-gene-related peptide promotes corneal epithelial migration in organ culture.

We have now shown that the combination of SP and IGF-1 promotes corneal epithelial wound healing in rats with trigeminal denervation. Given that SP alone does not stimulate epithelial migration in corneal organ culture, it is likely that both SP and IGF-1 are also essential in the promotion of epithelial migration in rats with corneal denervation. The combination of SP and IGF-1 thus stimulates epithelial migration in both innervated and denervated corneas. We selected the absolute concentrations and ratio of SP and IGF-1 and applied eye drops six times daily on the basis of the results of our previous in vitro and in vivo studies. The administration of eye drops containing both SP and IGF-1 has also been shown to be effective in the treatment of humans with neurotrophic or anhidrotic keratopathy. Similar treatment with the SP-derived peptide phenylalanine-glycine-leucine-methionine amide (FGLM-NH2) and IGF-1 exhibited a beneficial effect on superficial punctate keratitis in individuals with neurotrophic keratopathy.

Sigelman and Friedenwald described destruction of the ophthalmic branch of the trigeminal nerve by diathermy-induced coagulation in rats. In our adaptation of this procedure, we performed thermocoagulation of the ophthalmic nerve by inserting the electrode through the top of the skull rather than through the mouth. In our approach, the position of the trigeminal nerve is readily identifiable with the use of the bregma as a landmark, and the electrode can be fixed with a stereotaxic frame. It resulted in the loss of corneal sensation and an accompanying degeneration of the trigeminal nerve in 80% of eyes so treated. In addition to a loss of the blinking reflex, trigeminal denervation by our approach resulted in a significant decrease in the Schirmer test measurement. Given that the reflex secretion of tear fluid decreases secondarily to loss of theafferent fibres of the blinking reflex in the clinical setting, the reduction in the volume of tear fluid induced by trigeminal denervation may be due to the absence of reflex secretion.

By measuring fluorescein concentration in the anterior chamber with the use of a fluorescein fluorophotometer, Beuerman and Schimmelpfennig showed that corneal epithelial permeability increases 4 days after corneal sensory denervation in rabbits. In our rat model of neurotrophic keratopathy, the fluorescein permeability of the corneal epithelium as measured with an anterior fluorophotometer was significantly increased between 3 and 28 days after trigeminal denervation. Degeneration of the trigeminal nerve in our animal model also resulted in a delay in corneal epithelial wound healing, as previously observed in rabbits subjected to trigeminal denervation by thermocoagulation or resection. However, Sigelman and Friedenwald reported that trigeminal denervation by thermocoagulation did not induce a delay in corneal epithelial wound healing in rats. This apparent discrepancy with our results may be attributable to the difference in the manner of infliction of the epithelial wound. Whereas we removed the entire corneal epithelium with a dull blade, the epithelium was wounded by pinpricking in the previous study. Our rat model thus exhibits features of neurotrophic keratopathy in humans.

Corneal hypesthesia is often manifest in individuals with ocular herpetic infection, diabetes mellitus, or intraocular lesions or in those who abuse topical anesthetics. This condition may progress to superficial punctate keratitis, persistent epithelial defects, and, ultimately, stromal ulceration or perforation. A treatment for these disorders that is based on the pathobiology of neurotrophic keratopathy has been needed. The animal model described in the present study may both provide insight into the pathogenesis of corneal epithelial damage associated with neurotrophic keratopathy and facilitate the development of new therapies for corneal neurotrophic disorders.

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References

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