

Role of the Neurokinin-1 Receptor in the Promotion of Corneal Epithelial Wound Healing by the Peptides FGLM-NH₂ and SSSR in Neurotrophic Keratopathy

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Received: January 22, 2020

Accepted: June 19, 2020

Published: July 22, 2020

Citation: Yanai R, Nishida T, Hatano M, Uchi S-H, Yamada N, Kimura K. Role of the neurokinin-1 receptor in the promotion of corneal epithelial wound healing by the peptides FGLM-NH₂ and SSSR in neurotrophic keratopathy. *Invest Ophthalmol Vis Sci.* 2020;61(8):29. <https://doi.org/10.1167/iovs.61.8.29>

PURPOSE. Neurotrophic keratopathy is a corneal epitheliopathy induced by trigeminal denervation that can be treated with eyedrops containing the neuropeptide substance P (or the peptide FGLM-NH₂ derived therefrom) and insulin-like growth factor 1 (or the peptide SSSR derived therefrom). Here, we examine the mechanism by which substance P (or FGLM-NH₂) promotes corneal epithelial wound healing in a mouse model of neurotrophic keratopathy.

METHODS. The left eye of mice subjected to trigeminal nerve axotomy in the right eye served as a model of neurotrophic keratopathy. Corneal epithelial wound healing was monitored by fluorescein staining and slit-lamp examination. The distribution of substance P, neurokinin-1 receptor (NK-1R), and phosphorylated Akt was examined by immunohistofluorescence analysis. Cytokine and chemokine concentrations in intraocular fluid were measured with a multiplex assay.

RESULTS. Topical administration of FGLM-NH₂ and SSSR promoted corneal epithelial wound healing in the neurotrophic keratopathy model in a manner sensitive to the NK-1R antagonist L-733,060. Expression of substance P and NK-1R in the superficial layer of the corneal epithelium decreased and increased, respectively, in model mice compared with healthy mice. FGLM-NH₂ and SSSR treatment suppressed the production of interleukin-1 α , macrophage inflammatory protein 1 α (MIP-1 α) and MIP-1 β induced by corneal epithelial injury in the model mice. It also increased the amount of phosphorylated Akt in the corneal epithelium during wound healing in a manner sensitive to prior L-733,060 administration.

CONCLUSIONS. The substance P–NK-1R axis promotes corneal epithelial wound healing in a neurotrophic keratopathy model in association with upregulation of Akt signaling and attenuation of changes in the cytokine-chemokine network.

Keywords: corneal epithelium, substance P, neurokinin-1 receptor, insulin-like growth factor 1, persistent epithelial defect, neurotrophic keratopathy, Akt

The corneal epithelium forms a tight barrier that contributes to the transparency of the cornea and is maintained by the high turnover of corneal epithelial cells. As a result of the avascularity of the cornea, homeostasis and wound healing in the corneal epithelium are regulated by both neural factors derived from sensory nerves and humoral factors derived from tear fluid. We have previously shown that the neuropeptide substance P (SP) and insulin-like growth factor 1 (IGF-1) synergistically promote both corneal epithelial migration *in vitro*¹ and the closure of corneal epithelial wounds *in vivo* in both animals² and humans³ with neurotrophic keratopathy. These effects of SP and IGF-1 can be mimicked by a four-amino-acid peptide (Phe-Gly-Leu-Met-amide, or FGLM-NH₂) corresponding to the COOH-terminal region of SP and a four-amino-acid peptide (Ser-Ser-Ser-Arg, or SSSR) derived from the C domain of IGF-1.^{4–10}

Substance P is a member of the tachykinin family of neuropeptides that exerts its biological effects through interaction with the high-affinity neurokinin-1 receptor (NK-1R). The cornea is innervated by dense nerve endings of the trigeminal nerve, with some of these nerve fibers having been shown to contain SP.^{1,11–15} In addition to its role as a sensory neuropeptide, SP has been implicated in the regulation of homeostasis, inflammation, and neovascularization, as well as wound healing in the cornea.¹⁶ Moreover, NK-1R contributes to the maintenance of corneal epithelial homeostasis; its loss has been found to result in reduced tear volume, nerve density, and number of resident dendritic cells.¹⁷

Neurotrophic keratopathy is a degenerative disease of the cornea that results from denervation of the trigeminal nerve.^{4,5,18–20} It is characterized histologically by defective differentiation of corneal epithelial cells and delayed heal-

ing of corneal epithelial wounds.¹⁰ There is no specific clinical sign of neurotrophic keratopathy, for which the clinical manifestations include superficial punctate keratopathy, epithelial erosion, and persistence of epithelial defects.²¹ Clinical causes of the loss or impairment of trigeminal nerve function and consequent reduced corneal sensation include herpes simplex virus infection or herpes zoster ophthalmicus; complications of surgery for acoustic neuroma or of ophthalmic surgeries such as penetrating keratoplasty, LASIK, and photorefractive keratectomy; toxic keratopathy such as that resulting from abuse of topical anesthetics or β -blockers; and congenital diseases such as Riley-Day syndrome.^{19,21}

To develop eyedrops containing FGLM-NH₂ and SSSR for the treatment of persistent corneal epithelial defects in individuals with neurotrophic keratopathy, we previously examined the clinical efficacy of such eyedrops in a prospective single-center open clinical study.⁶ The eyedrops induced the rapid healing of such epithelial defects; however, the molecular mechanism of this effect has remained unclear. We have therefore now investigated the mechanism underlying the effect of the combination of FGLM-NH₂ and SSSR on corneal epithelial wound healing in neurotrophic keratopathy with the use of a mouse model of this condition.

METHODS

Animals and Materials

Six-week-old male Balb/c mice were obtained from Chiyoda Kaihatsu (Tokyo, Japan). Animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the study was approved by the Animal Care and Use Committee of Yamaguchi University (approval no. 41-032). Synthetic SSSR and FGLM-NH₂ peptides were synthesized by the Peptide Institute (Osaka, Japan), and the NK-1R antagonist L-733,060 was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Neurotrophic Keratopathy Model

A previously described mouse model based on trigeminal axotomy²² was adopted for the present study. This model manifests immediate loss of sub-basal nerve fibers in the axotomized eye and a delayed reduction in the number of such nerve fibers in the contralateral eye not subjected to axotomy. We therefore performed the surgical procedure in the right eye and studied the left eye as a model of neurotrophic keratopathy. In brief, mice were anesthetized by intraperitoneal injection of a mixture of ketamine (90 mg/kg; Daiichi Sankyo, Tokyo, Japan) and xylazine (10 mg/kg; Bayer Yakuhin, Osaka, Japan), and the ciliary fibers of the trigeminal nerve entering the sclera at the posterior globe were axotomized between the sclera and ciliary ganglion in the right eye. Control mice were not subjected to trigeminal axotomy but were otherwise maintained in a manner identical to that for the model mice. Corneal epithelial defect occurred in some mice; corneal neovascularization was observed in the axotomized eyes, and mild superficial epitheliopathy was observed in the untreated contralateral eyes of model mice. The left eyes of both control and model mice (14 days after surgery for the latter) were subjected to epithelial scraping, with or without subconjunctival injection of the NK-1R antagonist L-733,060 (6.6 μ g in 5 μ L of distilled

water) or distilled water alone 24 hours before scraping (Fig. 1).

Corneal Epithelial Wound Healing

Healthy control mice and neurotrophic keratopathy model mice were anesthetized with ketamine-xylazine as described above, the corneal epithelium of the left eye was demarcated with a 1-mm trephine, and a 1-mm circular cellulose sponge soaked in 70% ethanol was applied for 10 seconds. The epithelium was then removed within the circle with a small scalpel, leaving an intact basement membrane. Immediately and then 4, 8, 12, 16, 20, and 24 hours after the injury, either 5 μ L of a mixture of 1-mM FGLM-NH₂ and 100-nM SSSR in Ca²⁺- and Mg²⁺-free PBS, referred to as PBS(-), or of PBS(-) alone for the neurotrophic keratopathy model mice or 5 μ L of PBS(-) for the control mice was applied with a pipette. None of the treated eyes showed any sign of infection. The corneal epithelial defects were visualized at 0, 12, and 24 hours after injury by instillation of 0.25% fluorescein sodium and were photographed under examination with a slit-lamp microscope (SL-2G Slit Lamp; TOPCON, Tokyo, Japan) (Fig. 1). The stained area was measured with the use of ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Immunofluorescence Staining of Whole-Mount Preparations

The normal corneas of healthy mice and the left corneas of neurotrophic keratopathy model mice killed 14 days after axotomy in the right eye were excised, washed in PBS(-), and fixed in acetone for 15 minutes at room temperature. The corneas were then incubated for 90 minutes at room temperature in PBS(-) containing 3% BSA in order to block nonspecific staining before exposure (overnight at 4°C) to primary antibodies in PBS(-) containing 3% BSA. The antibodies included NL637-conjugated monoclonal anti- β III-tubulin (1:100 dilution; R&D Systems, Minneapolis, MN, USA), the polyclonal SP antibody N-18 (1:50 dilution, sc-9758; Santa Cruz Biotechnology, Dallas, TX, USA), and the polyclonal NK-1R antibody (1:50 dilution, NB300-101; Novus Biologicals, Centennial, CO, USA). The tissue was washed three times for 5 minutes with PBS(-), incubated for 1 hour at 4°C with Alexa Fluor 488 conjugated secondary antibodies (Thermo Fisher Scientific, Waltham, MA, USA) at a 1:2000 dilution in PBS containing 3% BSA, and then washed again. Corneal whole-mounts were prepared with VECTASHIELD mounting medium containing propidium iodide (PI; Vector Laboratories, Burlingame, CA, USA). Central and peripheral nerves of the whole-thickness cornea were imaged in z-axis steps of 2 mm with a laser confocal microscope (LSM Pascal; Carl Zeiss Meditec, Jena, Germany).

Immunofluorescence Staining of Corneal Sections

The normal corneas of healthy mice and the left corneas of neurotrophic keratopathy model mice killed 12 hours after epithelial scraping and treatment onset were excised, washed with PBS(-), fixed in 4% paraformaldehyde for 30 minutes at room temperature, washed again with PBS(-), and then embedded in optimum cutting temperature compound (Thermo Fisher Scientific). Cryosections of the cornea were prepared at a thickness of 6 μ m,

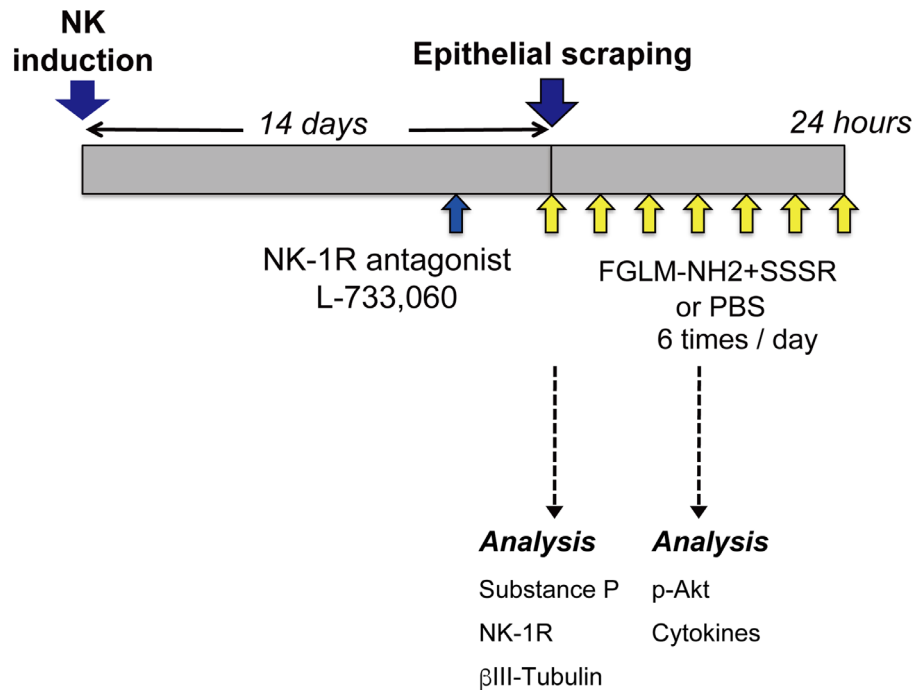


FIGURE 1. Experimental design. Neurotrophic keratopathy model mice were subjected to corneal epithelial scraping in the left eye 14 days after axotomy in the right eye. The mice were injected subconjunctivally in the left eye with the NK-1R antagonist L-733,060 or distilled water 24 hours before epithelial scraping, and the left eye was treated with eyedrops containing FGLM-NH₂ + SSSR peptides or PBS(-) vehicle immediately and then 4, 8, 12, 16, 20, and 24 hours after scraping. The expression of SP, NK-1R, and β III-tubulin in the left cornea was examined 14 days after contralateral axotomy, and the distribution of phosphorylated Akt (p-Akt) in the cornea and the concentrations of cytokines and chemokines in intraocular fluid were examined at 12 hours after epithelial scraping.

washed with PBS(-), permeabilized with methanol for 15 minutes at -20°C , and then incubated for 30 minutes at room temperature with 3% BSA in PBS(-) in order to block nonspecific staining. The sections were stained for 1 hour at room temperature with polyclonal antibodies to the Ser⁴⁷³-phosphorylated form of Akt (9271S; Cell Signaling Technology, Danvers, MA, USA) at a dilution of 1:100 in PBS(-) containing 3% BSA, washed with PBS(-), and then incubated for 1 hour at room temperature with Alexa Fluor 488 conjugated secondary antibodies at a dilution of 1:1000 in PBS(-) containing 3% BSA. They were washed again with PBS(-) before confocal fluorescence imaging with a BZ-X700 All-in-One Microscope (Keyence Corporation, Osaka, Japan) equipped with a 40 \times objective and 530-nm excitation and 615-nm emission filters.

Analysis of Cytokines and Chemokines

The normal eyeballs of healthy mice and the left eyeballs of neurotrophic keratopathy model and control mice killed 12 hours after epithelial scraping and treatment onset were excised, trimmed of extraocular muscles and soft tissue, and then crushed with the use of a BioMasher (Sarstedt, Nürnbrecht, Germany). The crushed tissue was centrifuged at 9000g for 30 seconds, and the resulting supernatant (intraocular fluid) was stored at -80°C until assay of cytokine and chemokine concentrations with the use of a Bio-Plex Pro Mouse Cytokine 23-Plex Panel and Bio-Plex Manager software version 4.1.1 (Bio-Rad, Hercules, CA, USA).

Reverse Transcription and Real-Time Polymerase Chain Reaction Analysis

The normal corneas of healthy mice and the left corneas of neurotrophic keratopathy model mice killed 14 days after axotomy in the right eye were excised, washed in PBS(-), and pooled separately ($n = 4$ or 5) to reduce biological variability. Total RNA was isolated from the cornea tissue with the use of an RNeasy Mini Kit (Qiagen, Hilden, Germany) and was subjected to reverse transcription (RT) with a Promega (Madison, WI, USA) reverse transcription system. The resulting cDNA was subjected to real-time PCR analysis with the use of PowerUp SYBR Green Master Mix (Thermo Fisher Scientific) and a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The PCR primers (forward and reverse, respectively) were 5'-GACAGTGACCA GATCAAGGAG-3' and 5'-GCTTGCCCATTAATCCAAAGAAC-3' for SP, 5'-AGAACATCCCAACAGGACTTAC-3' and 5'-AGTGTAATCCCTACCACAGTGTAT-3' for NK-1R, and 5'-AGCCTCAAGATCATCAGCAAT-3' and 5'-CCTTCCACGATACCAAAGTTGT-3' for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The fold changes in gene expression levels were calculated by the delta delta C_t method. The amounts of SP and NK-1R mRNAs were normalized by that of GAPDH mRNA.

Immunoblot Analysis

The normal corneas of healthy mice and the left corneas of neurotrophic keratopathy model mice killed 12 hours after epithelial scraping and treatment onset were excised, washed in PBS(-), and pooled separately ($n = 4$ or 5) to

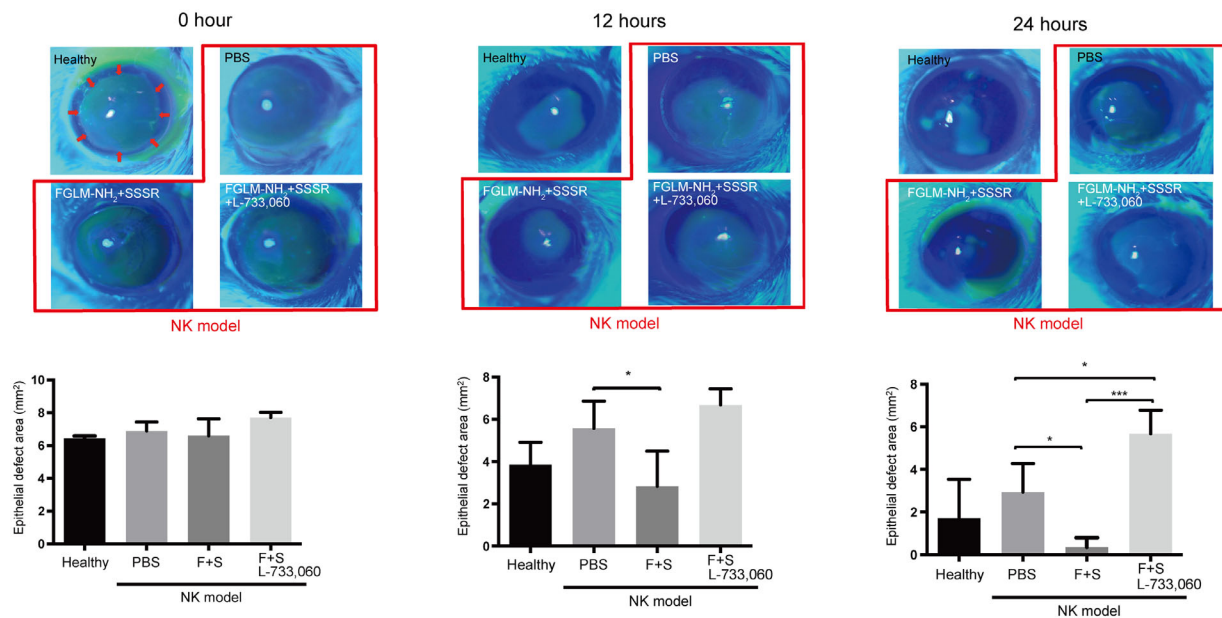


FIGURE 2. The combination of FGLM-NH₂ and SSSR peptides promotes corneal epithelial wound healing in an L-733,060-sensitive manner in neurotrophic keratopathy. The healing of corneal epithelial wounds in healthy mice as well as in neurotrophic keratopathy model mice treated with PBS(-) vehicle or FGLM-NH₂ + SSSR (F + S) peptides (with or without prior subconjunctival injection of the NK-1R antagonist L-733,060) was examined by staining with fluorescein sodium at 0, 12, and 24 hours after epithelial scraping. Representative slit-lamp photographs and quantitative data (means + SD) for three, five, four, and five mice in the healthy, PBS(-), FGLM-NH₂ + SSSR, and FGLM-NH₂ + SSSR + L-733,060 groups, respectively, are shown. **P* < 0.05, ****P* < 0.001 (Tukey–Kramer test).

reduce biological variability. The corneal tissue was lysed in 300 μ L of a solution containing 1% Nonidet P-40, 50-mM Tris-HCl (pH 7.4), 100-mM NaCl, 10-mM MgCl₂, 1-mM dithiothreitol, 1-mM phenylmethylsulfonyl fluoride, and 1% protease inhibitor cocktail (all, Sigma-Aldrich). The lysates were then centrifuged at 20,000g for 5 minutes at 4°C, and the resulting supernatants were stored at -80°C until analysis. The supernatants (20 μ g of protein) were subsequently fractionated by SDS-PAGE on a 10% gel, the separated proteins were transferred electrophoretically to a nitrocellulose membrane, and nonspecific sites of the membrane were blocked before incubation with primary antibodies (each at a 1:1000 dilution) to Ser⁴⁷³-phosphorylated (9271S) or total (9272) forms of Akt (Cell Signaling Technology). Immune complexes were detected with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescent reagents (GE Healthcare, Little Chalfont, UK).

Statistical Analysis

Quantitative data are presented as means \pm SD or SEM and were analyzed with Student's *t*-test or the Tukey–Kramer multiple comparison test. *P* < 0.05 was considered statistically significant.

RESULTS

FGLM-NH₂ Promotes Corneal Epithelial Wound Healing in a Manner Dependent on NK-1R

To study the effect of SP on corneal epithelial wound healing in a mouse model of neurotrophic keratopathy, we treated eyes with a combination of FGLM-NH₂ and SSSR peptides over a 24-hour period after epithelial scraping (Fig. 1). The possible role of NK-1R in the action of SP was examined by

subconjunctival injection of the NK-1R antagonist L-733,060 24 hours before epithelial scraping and treatment onset. Immediately after corneal epithelial scraping, the size of the epithelial defect was similar in each group of mice: (1) healthy mice (6.437 \pm 0.148 mm²); (2) neurotrophic keratopathy model mice treated with PBS(-) vehicle (6.889 \pm 0.550 mm²); (3) neurotrophic keratopathy model mice treated with FGLM-NH₂ + SSSR + L-733,060 (7.700 \pm 0.329 mm²); and (4) neurotrophic keratopathy model mice treated with FGLM-NH₂ + SSSR but not L-733,060 (6.594 \pm 1.034 mm²) (Fig. 2). Twelve hours after epithelial scraping, the epithelial defect in the FGLM-NH₂ + SSSR group (2.832 \pm 1.662 mm²) was significantly smaller than that in the PBS(-) group (5.578 \pm 1.288 mm²) of neurotrophic keratopathy model mice. At 24 hours after injury, the epithelial defect was significantly smaller in the FGLM-NH₂ + SSSR group (0.352 \pm 0.443 mm²) than in both the PBS(-) group (2.936 \pm 1.342 mm²) and the FGLM-NH₂ + SSSR + L-733,060 group (5.672 \pm 1.110 mm²) of model mice. The epithelial defect in the FGLM-NH₂ + SSSR + L-733,060 group was also significantly larger than that in the PBS(-) group. These results suggest that the FGLM-NH₂ + SSSR eyedrops promoted corneal epithelial wound healing in a manner dependent on NK-1R in mice with neurotrophic keratopathy.

SP and NK-1R Expression in the Corneal Epithelium of Neurotrophic Keratopathy Model Mice

To confirm the loss of corneal nerve fibers in the mouse model of neurotrophic keratopathy, we performed immunofluorescence staining of whole-mount corneal preparations with antibodies to the neuron-specific protein

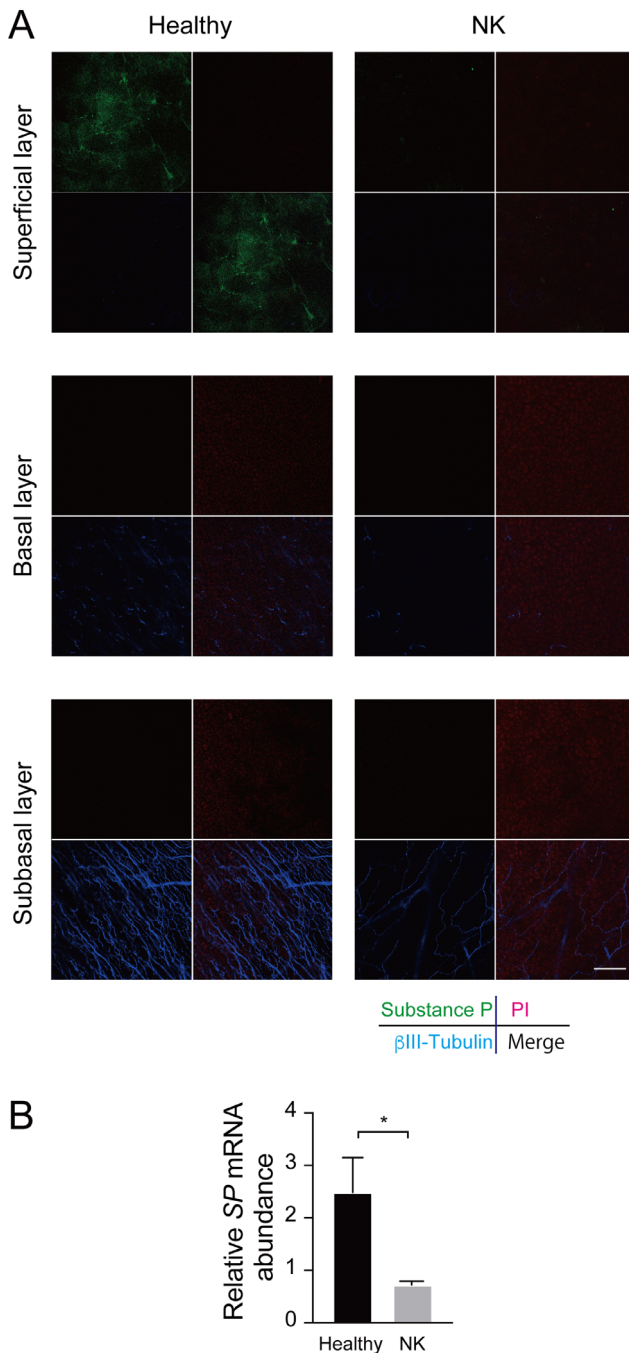


FIGURE 3. Reduced expression of SP in the corneal epithelium of neurotrophic keratopathy model mice. **(A)** Corneal whole-mount preparations from healthy mice and from neurotrophic keratopathy model mice at 14 days after axotomy in the contralateral eye were subjected to immunofluorescence staining of SP and β III-tubulin. Nuclei were stained with PI. Data are representative of those from a total of five mice per group. Bar: 50 μ m. **(B)** RT and real-time PCR analysis of relative SP mRNA abundance in the cornea of mice as in A. Data are means \pm SEM for X pooled samples each derived from four or five mice. * $P < 0.05$ (Student's *t*-test).

β III-tubulin and to SP. Trigeminal axotomy indeed reduced the density of corneal nerves in the sub-basal layer of the contralateral eyes compared with that apparent in healthy eyes (Fig. 3A; Supplementary Videos S1, S2). In addition, the extent of SP immunoreactivity in the superficial layer of the corneal epithelium was also reduced

in neurotrophic keratopathy model mice compared with healthy mice (Fig. 3A). Moreover, the amount of SP mRNA in the corneas of the neurotrophic keratopathy model mice was significantly reduced compared with that in the healthy corneas (Fig. 3B). These results suggest that the production and secretion of SP in the corneal epithelium were attenuated in the model mice.

We also examined expression of the SP receptor NK-1R in the cornea. Immunofluorescence for NK-1R in the superficial layer of the corneal epithelium was increased in the neurotrophic keratopathy model mice compared with healthy mice (Fig. 4A; Supplementary Videos S3, S4), suggesting that the expression of NK-1R in the corneal epithelium was upregulated in response to the depletion of SP in the model mice. However, the amount of NK-1R mRNA in the cornea was not increased in neurotrophic keratopathy model mice compared with healthy mice (Fig. 4B).

FGLM-NH₂ and SSSR Peptides Attenuate Production of IL-1 α and Macrophage Inflammatory Protein 1 α and 1 β During Corneal Epithelial Wound Healing in Neurotrophic Keratopathy

Nerves containing SP innervate primary and secondary lymphoid organs,^{23–25} suggesting that SP may serve as a mediator of cross-talk between the nervous and immune systems. To examine possible immune-related effects of SP during corneal epithelial wound healing in neurotrophic keratopathy model mice, we measured the concentrations of various cytokines and chemokines in intraocular fluid at 12 hours after epithelial scraping and treatment onset. Among 23 cytokines and chemokines examined, the concentrations of IL-1 α ($P \leq 0.0001$), macrophage inflammatory protein 1 α (MIP-1 α) ($P \leq 0.0001$), and MIP-1 β ($P \leq 0.0001$) were increased by scraping to a greater extent in neurotrophic keratopathy model eyes than in healthy eyes, and they were significantly reduced ($P < 0.0001$, $P < 0.01$, and $P < 0.01$, respectively) in the scraped model eyes that received treatment with FGLM-NH₂ + SSSR (Fig. 5). IL-1 α and MIP-1 α were not detected in healthy eyes or in eyes with neurotrophic keratopathy not subjected to epithelial scraping. Although the concentrations of various other cytokines or chemokines were affected by the induction of neurotrophic keratopathy or by epithelial scraping, none was changed by peptide treatment (Fig. 5). These results suggest that SP-mediated cross-talk between the nervous and immune systems might contribute to the promotion of corneal epithelial wound healing by SP.

FGLM-NH₂ Activates Akt in a Manner Dependent on NK-1R During Corneal Epithelial Wound Healing in Neurotrophic Keratopathy

To further investigate the mechanism underlying the promotion by SP of corneal epithelial wound healing in neurotrophic keratopathy, we examined the effect of FGLM-NH₂ and SSSR on the activation status of the protein kinase Akt in the corneal epithelium. Immunofluorescence staining of corneal sections (Fig. 6A) and immunoblot analysis of corneal tissue lysates (Fig. 6B) revealed that the abundance of phosphorylated (activated) Akt at 12 hours after epithelial scraping was increased by treatment with FGLM-NH₂ + SSSR eyedrops. Furthermore, this effect of the

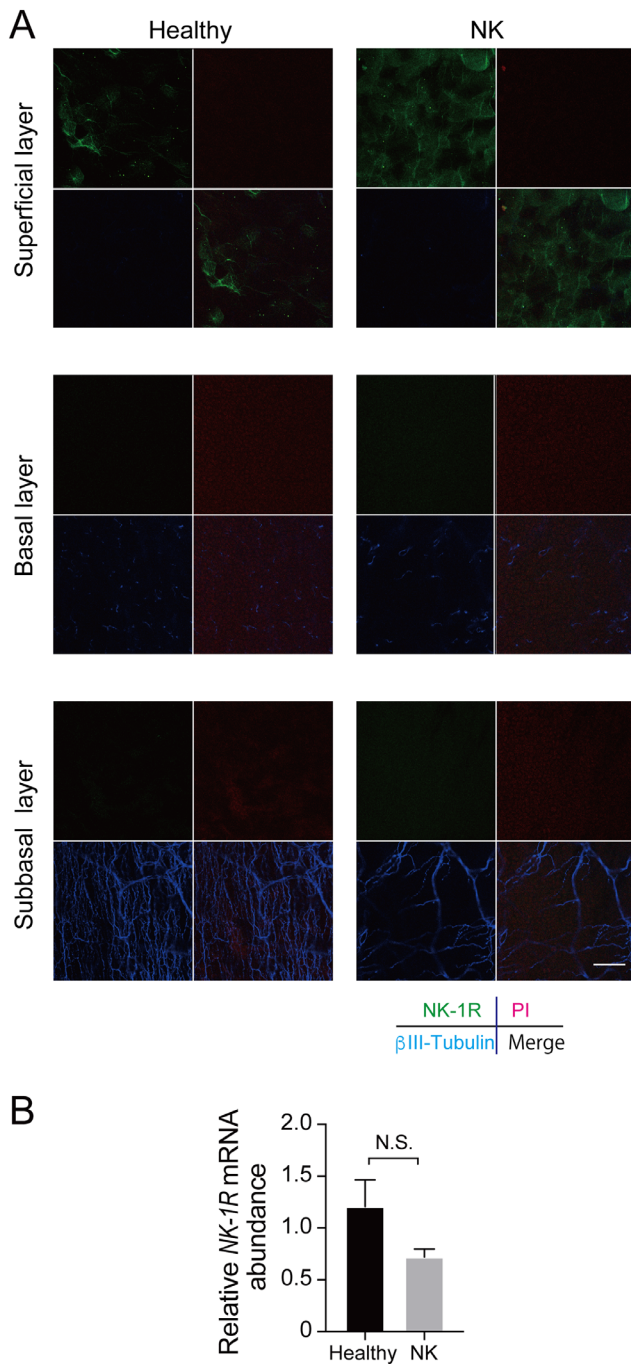


FIGURE 4. Increased expression of NK-1R in the corneal epithelium of neurotrophic keratopathy model mice. **(A)** Corneal whole-mount preparations from healthy mice and from neurotrophic keratopathy model mice at 14 days after axotomy in the contralateral eye were subjected to immunofluorescence staining of NK-1R and β III-tubulin. Nuclei were stained with PI. Data are representative of those from a total of five mice per group. Bar: 50 μ m. **(B)** RT and real-time PCR analysis of relative NK-1R mRNA abundance in the cornea of mice as in **A**. Data are means \pm SEM for X pooled samples each derived from four or five mice. N.S., not significant (Student's *t*-test).

peptide eyedrops was prevented by prior subconjunctival injection of the NK-1R-specific antagonist L-733,060. These results thus suggest that FGLM-NH₂ activated Akt via NK-1R during corneal epithelial wound healing in neurotrophic keratopathy.

DISCUSSION

We have shown here that the SP-derived peptide FGLM-NH₂ and the IGF-1-derived peptide SSSR promoted corneal epithelial wound healing in a mouse model of neurotrophic keratopathy in a manner dependent on NK-1R. We also found that the expression of NK-1R was upregulated in the superficial layer of the corneal epithelium in this mouse model. Furthermore, FGLM-NH₂ + SSSR eyedrops activated Akt in the corneal epithelium in a manner dependent on NK-1R and suppressed inflammatory cytokine and chemokine production during corneal epithelial wound healing in the model mice. Together, these results suggest that the SP-NK-1R axis promotes corneal epithelial wound healing in mice with neurotrophic keratopathy and that the activation of Akt signaling and modulation of the local cytokine-chemokine network may contribute to this effect.

Various therapeutic approaches have been developed to facilitate corneal epithelial healing and to prevent the occurrence or recurrence of epithelial breakdown, as well as corneal stromal lysis and perforation in patients with neurotrophic keratopathy.^{4,5,20} However, the management of this condition remains a challenge, and the development of new therapeutic options is therefore desirable.

We previously showed that SP or FGLM-NH₂ modulates corneal epithelial migration by interacting with NK-1R, thereby stimulating the phospholipase C-mediated production of inositol 1,4,5-trisphosphate, the release of Ca²⁺ from intracellular stores, and the activation of Ca²⁺- and calmodulin-dependent protein kinase II.^{9,26} The combination of SP (or FGLM-NH₂) and IGF-1 (or SSSR) promotes corneal epithelial wound healing *ex vivo*, in animals, and in patients with neurotrophic keratopathy.¹⁻⁸ In the present study, we found that FGLM-NH₂ + SSSR eyedrops promoted corneal epithelial wound healing in a mouse model of neurotrophic keratopathy. This effect of FGLM-NH₂ + SSSR is consistent with that previously observed in healthy rabbits.² Furthermore, we found that the reduced density of nerve fibers in the sub-basal layer was accompanied both by both loss of SP and by upregulation of NK-1R in the superficial layer of the corneal epithelium of mice with neurotrophic keratopathy.

SP is a member of the tachykinin family of neuropeptides that share common pharmacological properties and a conserved COOH-terminal sequence (Phe-X-Gly-Leu-Met-NH₂, where X is a hydrophobic or aromatic amino acid).²⁷ Tachykinins are expressed widely throughout the nervous and immune systems. The major mammalian tachykinins are SP, neurokinin A, neurokinin B, neuropeptide K, and neuropeptide γ ; of these, SP in particular has been shown to regulate cytokine release by various cell types and is implicated in modulation of immune responses at peripheral sites—such as the gastrointestinal and respiratory tracts and corneal epithelium—at which the extent of inflammation correlates with that of NK-1R activation.^{8,28,29} Impairment of SP-NK-1R signaling in the corneal epithelium may contribute to the attenuation of corneal sensitivity and loss of corneal epithelial homeostasis in diabetes.³⁰ The SP-NK-1R axis is thus a promising target for the treatment of neurotrophic keratopathy.

Proinflammatory cytokines such as IL-1 α and chemokines such as MIP-1 α and MIP-1 β play important roles in corneal epithelial pathophysiology such as those associated with alkali burns, trauma, corneal graft rejection, and viral infection.³¹ Cytokines and chemokines released by

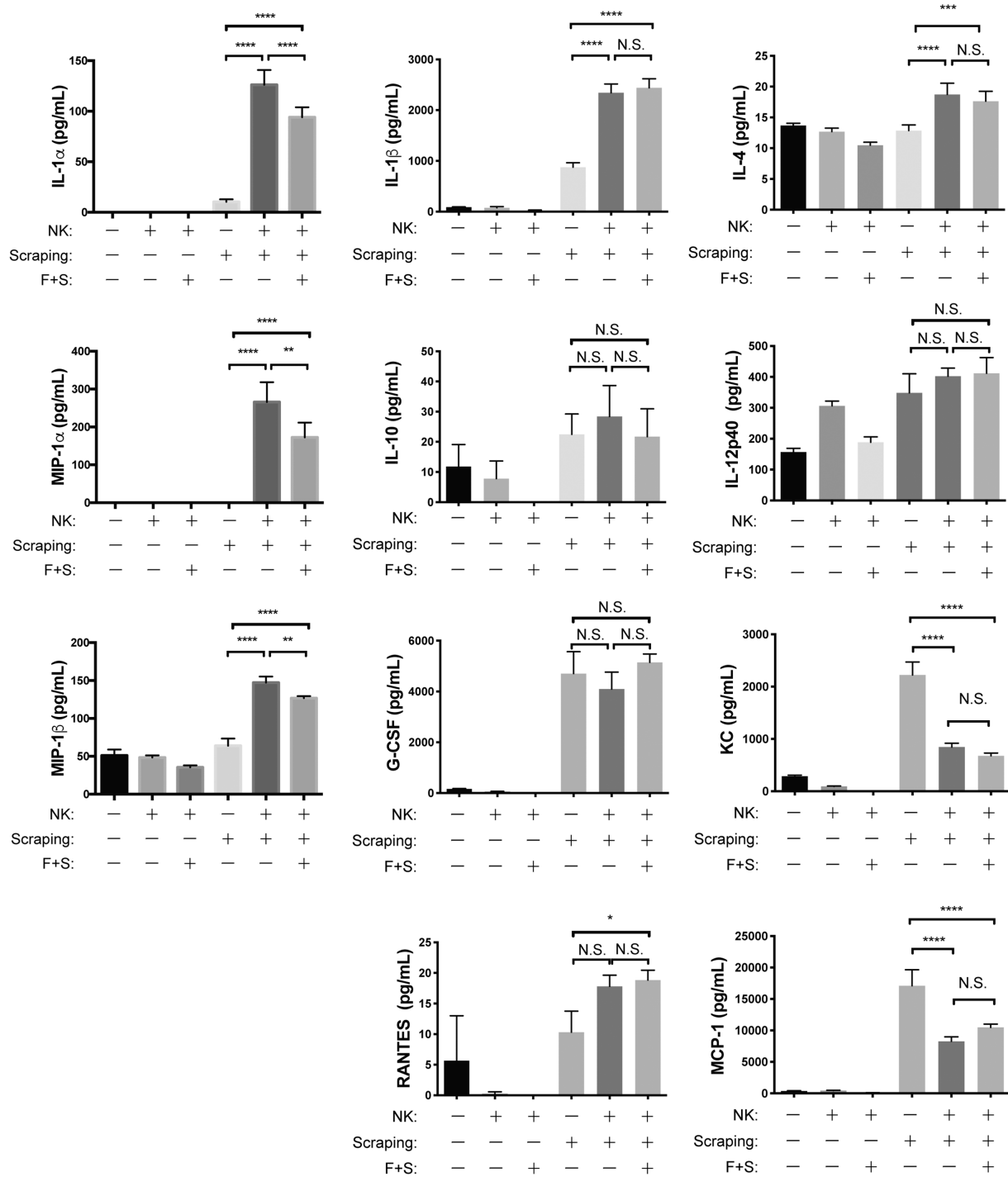


FIGURE 5. Concentrations of IL-1 α , MIP-1 α , MIP-1 β , and various other cytokines and chemokines in intraocular fluid during corneal epithelial wound healing in healthy mice or in neurotrophic keratopathy model mice treated with FGLM-NH₂ and SSSR. The concentrations were measured with a multiplex assay 12 hours after corneal epithelial scraping and the onset of treatment with FGLM-NH₂ + SSSR (F + S) eyedrops or vehicle in healthy or neurotrophic keratopathy model mice. IL-2, IL-3, IL-5, IL-9, IL-10, IL-12p70, IL-13, IL-17A, eotaxin, granulocyte-macrophage colony-stimulating factor, interferon γ , and tumor necrosis factor α were not detected. Data are means \pm SEM for four pooled samples each derived from four mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. N.S., not significant (Tukey-Kramer test); G-CSF, granulocyte colony-stimulating factor; KC, keratinocyte chemoattractant; RANTES, regulated on activation normal T expressed and secreted; MCP-1, monocyte chemoattractant protein 1.

tissue-resident cells and infiltrating immune cells contribute to local immune responses and inflammation and are thought to delay corneal epithelial wound healing in neurotrophic keratopathy.^{11,20} We have now shown that

FGLM-NH₂ + SSSR eyedrops suppress the production of IL-1 α , MIP-1 α , and MIP-1 β induced by corneal epithelial scraping in mice with neurotrophic keratopathy, suggesting that these peptides facilitate corneal epithelial wound healing at

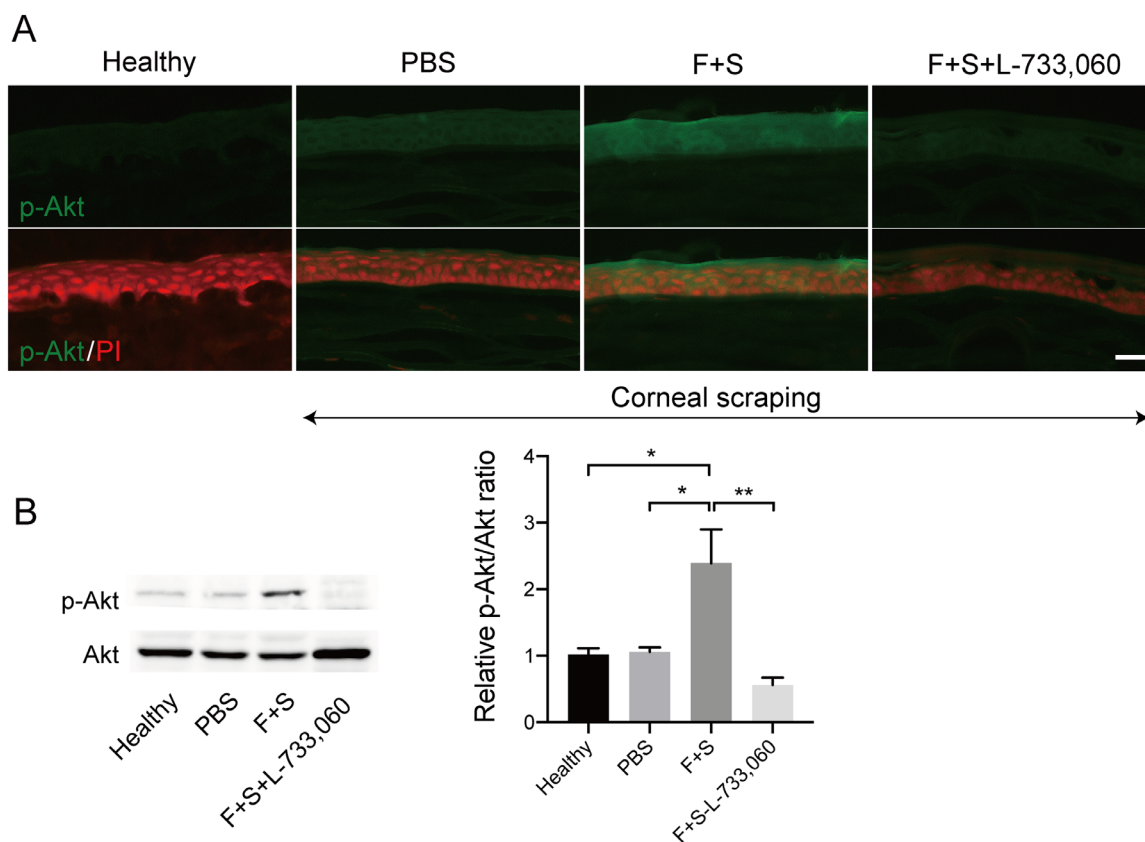


FIGURE 6. FGLM-NH₂ + SSSR treatment activates Akt in a manner dependent on NK-1R during corneal epithelial wound healing in neurotrophic keratopathy. **(A)** Corneal sections prepared from a healthy eye or from neurotrophic keratopathy model eyes at 12 hours after epithelial scraping and the onset of treatment with FGLM-NH₂ + SSSR (F+S) or PBS(-) vehicle, with or without subconjunctival injection of L-733,060 24 hours before scraping, were subjected to immunofluorescence staining with antibodies to p-Akt. Nuclei were stained with PI. Data are representative of those from a total of five mice per group. Bar: 20 μ m. **(B)** Corneal tissue lysates prepared from mice as in **A** were subjected to immunoblot analysis with antibodies to total or phosphorylated forms of Akt. A representative immunoblot and quantitative data (means \pm SEM) for densitometric determination of the relative p-Akt/Akt ratio for *X* pooled samples each derived from four or five mice are shown. * $P \leq 0.05$, ** $P \leq 0.01$ (Tukey-Kramer test).

least in part by modulating the local immune-inflammatory environment.

SP was shown to enhance cell proliferation and to attenuate or delay apoptosis in keratocytes both in vitro and in vivo³² in a manner dependent on epidermal growth factor receptor-Akt signaling. Moreover, SP stimulated the phosphorylation of Akt in association with promotion of corneal epithelial wound healing in mice with diabetes-related neurotrophic keratopathy.³⁰ We have now shown that FGLM-NH₂ + SSSR eyedrops can induce the phosphorylation (activation) of Akt in the corneal epithelium during epithelial wound healing in mice with neurotrophic keratopathy, suggesting that activation of Akt signaling might contribute to the promotion of corneal epithelial wound healing by these peptides. Sensory neurons have been shown to play a prominent role in regulation of immune responses and inflammation,³³⁻³⁵ and the possible effects of SP on the function of innate and adaptive immune cells and on tissue-resident cells and the vasculature in the healthy and neurotrophic corneas warrant further investigation.

Neurotrophic keratopathy is characterized by a reduction in corneal sensitivity, spontaneous breakdown of the corneal epithelium, and impairment of corneal healing.^{17,19,36,37} Persistent corneal epithelial defects (PEDs) associated with

neurotrophic keratopathy are difficult to treat because of the loss of neural regulation, deficiency of neurotrophic factors, and hypolacrimation in affected individuals. In its most severe form, neurotrophic keratopathy can lead to loss of vision as a result of corneal ulceration and perforation. Forced eye patches, therapeutic soft contact lenses, oily ointments, tarsorrhaphy, and amniotic membrane transplantation have been applied to the treatment of PEDs in an attempt to improve the environment at the ocular surface. Recently, the U.S. Food and Drug Administration approved Oxervate (cenegermin-bkbj), a topical formulation of nerve growth factor, as a treatment to promote the healing of corneal ulcers associated with neurotrophic keratopathy.^{17,38-41} However, new drugs for the treatment of neurotrophic keratopathy are still needed, given that the use of Oxervate is restricted in some patients because of adverse reactions, including eye pain, ocular hyperemia, eye inflammation, and increased lacrimation.

In the present study, we examined the left eye of mice subjected to trigeminal axotomy in the right eye as a model of neurotrophic keratopathy in order to avoid technical variability due to the surgical procedure. However, sympathetic neurogenic inflammation might affect wound healing and immune processes in both eyes, as the severing of corneal nerves in one eye have been found to induce not only the

loss of corneal nerve fibers but also the loss of immune privilege as a result of downregulation of CD103 on T regulatory cells in both eyes.^{42,43} Contralateral effects have also been described in patients with unilateral herpes simplex keratitis⁴⁴ or glaucoma (diurnal fluctuation of intraocular pressure),⁴⁵ as well as in a unilateral model of capsaicin-induced neurogenic inflammation.⁴⁶ The mechanisms of such contralateral effects in unilateral conditions remain unclear, but such phenomena have also been observed in numerous experimental and clinical paradigms outside the eye.^{47–52}

In summary, our results indicate that the SP-derived peptide FGLM-NH₂ interacts with NK-1R to promote corneal epithelial wound healing in a mouse model of neurotrophic keratopathy, and that both the activation of Akt and attenuation of the production of proinflammatory cytokines and chemokines may contribute to this effect. Topical cenergermin has recently been shown to promote the healing of PEDs in patients with neurotrophic keratopathy; however, this treatment did not achieve complete recovery or prevention of recurrence.^{11,53,54} Treatment with SP or FGLM-NH₂ in combination with IGF-1 or SSSR may address the deficits that underlie the pathophysiological features of neurotrophic keratopathy. Further studies are required to clarify the potential of such regimens or of other approaches that target the SP–NK-1R axis for the treatment of neurotrophic keratopathy.

Acknowledgments

The authors thank Yukari Mizuno and Ayaka Kataoka for their technical assistance.

Disclosure: **R. Yanai**, None; **T. Nishida**, FGLM-NH₂ + SSSR eyedrops (P); **M. Hatano**, None; **S.-H. Uchi**, None; **N. Yamada**, None; **K. Kimura**, None

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SUPPLEMENTARY MATERIAL

SUPPLEMENTARY VIDEO S1. Expression of SP in the corneal epithelium of neurotrophic keratopathy model mice.

SUPPLEMENTARY VIDEO S2. Expression of SP in the corneal epithelium of healthy mice.

SUPPLEMENTARY VIDEO S3. Expression of NK-1R in the corneal epithelium of neurotrophic keratopathy model mice.

SUPPLEMENTARY VIDEO S4. Expression of NK-1R in the corneal epithelium of healthy mice.