Corneal Epithelium-Derived Neurotrophic Factors Promote Nerve Regeneration

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PurPosE. To explore the neurotrophic factor expression in corneal epithelium and evaluate their effects on the trigeminal ganglion (TG) neurite outgrowth and corneal nerve regeneration in mice.

MethoDs. The expression of neurotrophic factors was compared among the intact, regenerating, and regenerated mouse corneal epithelium. Mouse primary TG neurons were treated with the conditioned medium of mouse corneal epithelial cells. Nerve growth factor (NGF) neutralizing antibody and glial cell-derived neurotrophic factor (GDNF) neutralizing antibody were used to evaluate their roles in mouse corneal nerve regeneration and TG neurite outgrowth. The promoting effects of NGF and GDNF for the corneal nerve regeneration were further evaluated in the diabetic mice.

ResuLtS. The expression of NGF and GDNF showed significant up-regulation in regenerating corneal epithelium and return to the preinjury levels in the regenerated epithelium, which was consistent with the progress of corneal subbasal nerve regeneration. The conditioned medium of corneal epithelial cells promoted the TG neurite outgrowth with extended branching and elongation. Furthermore, the blockage of either NGF or GDNF significantly impaired the promotion of the neurite outgrowth by the conditioned medium or the corneal nerve regeneration in normal mice. Moreover, the expression of NGF and GDNF was attenuated in the diabetic regenerating corneal epithelium as compared to that in normal mice, while exogenous NGF or GDNF supplement promoted the corneal epithelial and nerve regeneration in diabetic mice.

ConcluSioNs. Corneal epithelium expresses multiple neurotrophic factors, among which NGF and GDNF may play an important role in the corneal nerve regeneration.

Keywords: corneal epithelium, neurotrophic factors, nerve regeneration, diabetes

The cornea is the most densely innervated tissue in the human body and is predominantly supplied by sensory nerve fibers. The normal and proper corneal innervation is responsible for the maintenance of corneal sensation, blink reflex, ocular surface homeostasis, and also involved in the regulation of corneal wound healing. Therefore, the impairment of corneal innervation, due to for instance viral infection, trauma, or surgical procedures, can cause attenuated corneal sensitivity, dry eye, neurotrophic keratitis, and even blindness. In diabetic cornea, hyperglycemia can evoke impaired corneal sensitivity, attenuated nerve fiber density, and delayed epithelial wound healing, which has been hypothesized to be caused by the impairment of corneal innervation. Despite the high prevalence of corneal diseases and nerve dysfunction, there are relatively few therapeutic treatments of regulating corneal nerve functions and recovering corneal innervation for patients.

Corneal nerve fibers originate from the ophthalmic division of the trigeminal ganglion (TG), form subbasal nerve plexus underneath the basal epithelium, and terminate in the corneal epithelium in the forms of free terminals or synapse-like structures. Accumulating evidence suggests that corneal nerve fibers and corneal epithelium mutually support each other through the release of soluble trophic substances. Corneal nerve fibers release multiple neuromediators that maintain corneal epithelial homeostasis and stimulate wound healing once injured. Meanwhile, previous studies have demonstrated that conditioned medium from cultured corneal epithelial cells, but not corneal stromal fibroblasts or endothelial cells, prolongs neuronal survival and induces neurite outgrowth. More recently, multiple neurotrophic factors have been detected in limbal and corneal epithelium. However, the key factors derived from corneal epithelium and their essential roles during corneal nerve regeneration remain elusive.

Our previous study has confirmed that the neuropeptide substance P and the ciliary neurotrophic factor promote the corneal epithelial wound healing and nerve regeneration in mice. In the present study, we first examined the expression changes of several known neurotrophic factors in the corneal epithelium during the regeneration of corneal subbasal nerve fibers, and subsequently explored their effects in the nerve regeneration in vitro and in vivo. The results suggest that the expression of nerve growth factor (NGF) and glial cell-derived neurotrophic factor (GDNF) in corneal epithelium shows significant changes during corneal nerve...
fiber regeneration, and that the blockage of NGF or GDNF significantly impairs the corneal nerve regeneration in normal mice. In addition, we further confirmed the expression of NGF and GDNF was attenuated in the diabetic mouse corneal epithelium, and that exogenous NGF or GDNF supplement promoted the corneal nerve regeneration in diabetic mice.

**Materials and Methods**

**Animals**

Male C57BL/6 mice 6 to 8 weeks old were purchased from the Beijing Pharmacology Institute (Beijing, China) and maintained in the animal center of Shandong Eye Institute. All animal experiments were approved by the Ethics Committee of Shandong Eye Institute and carried out in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. Type 1 diabetic mice were induced with intraperitoneal injections of streptozotocin according to our previous description, and subsequently selected with the blood glucose level higher than 300 mg/dL after 12 weeks of the final injection for further experiments.

**Corneal Epithelial Wound Healing**

Mouse central corneal epithelium (diameter 2 mm) was removed with Algerbrush II corneal rust ring remover (Alger Co., Lago Vista, TX, USA) under anaesthesia. For growth factor neutralization, normal mice were injected subconjunctivally with 5 ng/mL L-3-isoform-matched IgG (R&D), or 5 ng/mL L-3-isoform-matched IgG (R&D) 24 hours before and 0 hour, and 24 hours after the removal of corneal epithelium, with the same concentration of isoform-matched IgG (R&D) injection for control mice. For growth factor treatment, diabetic mice were injected subconjunctivally with 50 ng NGF (5 μL/eye; Abcam, Cambridge, MA, USA) or 50 ng GDNF (5 μL/eye; R&D, San Diego, CA, USA) 24 hours before and 0 hour, and 24 hours after the removal of corneal epithelium, with the same concentration of isoform-matched IgG (R&D) injection for control mice. For growth factor treatment, diabetic mice were injected subconjunctivally with 50 ng NGF (5 μL/eye, R&D) or 50 ng GDNF (5 μL/eye, R&D) immediately after the epithelial removal, while the mice received PBS as vehicle control. The unhealed corneal epithelial defect was visualized by fluorescein staining and calculated as the percentage of original defect. All the dosages used in the present study had been evaluated and optimized in our primary experiments.

**Corneal Sensitivity Measurement**

Corneal sensitivity was measured according to our previous descriptions by using the Cochet-Bonnet esthesiometer (Luneau Ophthalmologie, Chartres Cedex, France) in unanesthetized diabetic mice after 7 days of NGF or GDNF treatment. The longest filament length with the positive response was considered as the corneal sensitivity threshold, which was verified at least four times.

**In Vitro Wound Scratch Assay**

Mouse corneal epithelial cell line (TKE2) was cultured in keratinocyte serum free medium (KSF; Invitrogen, Carlsbad, CA, USA) supplemented with bovine pituitary extract and epidermal growth factor. The cells were seeded into six-well tissue culture plates, cultured to confluence, and wounded with a 200-μL pipette tip for eight lanes. Following PBS washes, cultures were refreshed with basic KSF medium and incubated for 48 hours. The supernatant was centrifuged and applied as conditioned medium for the subsequent experiments. The conditioned medium from the unwounded confluent cells was used as control.

**TG Neuron Culture and Treatment**

TG neurons were isolated and dissociated from normal mice as described previously. In brief, the ophthalmic branches of TG were harvested and subjected to enzymatic digestion with papain and collagenase/dispace. The isolated cells were separated in Percoll gradients, plated onto poly-D-lysine and laminin-coated slides (Thermo Scientific, Waltham, MA, USA) and incubated in the neurobasal-A medium (Life Technologies, Grand Island, NY, USA) with B27 supplement (Life Technologies). After attachment for overnight, the neuron cells were refreshed with medium containing 50% neurobasal-A medium and 50% conditioned medium of mouse TKE2 cells for evaluating its effects on neurite outgrowth. For inhibitory studies, the cells were incubated with NGF neutralizing antibody (1:100; Abcam) or GDNF neutralizing antibody (1:100; R&D) for 48 hours in the presence of the conditioned medium. Neurite outgrowth was analyzed and calculated by using quantitative nerve tracing software Neuron J (a plugin for ImageJ, National Institutes of Health, Bethesda, MA, USA).

**Immunofluorescence Staining**

Cornea whole-mount staining was performed as previously described. In brief, mouse eyeballs were collected and fixed in Zamboni’s fixative for 1 hour with the cornea dissected around the scleral-limbal region at 48 hours after injury. The cornea was blocked by PBS with 0.1% Triton X-100, 2% goat serum, and 2% bovine serum albumin for 2 hours, and subsequently incubated in the same incubation buffer with Alexa Fluor 488 conjugated neuronal class III β-tubulin mouse monoclonal antibody (Merck Millipore, Darmstadt, Germany) overnight at 4°C. After washing for five times, the flat mounts were examined under an Eclipse TE2000-U microscope (Nikon, Tokyo, Japan) after counterstained with 4′,6-diamidino-2-phenylindole (DAP). To visualize the neurite outgrowth from TG, the cultured neurons were incubated with mouse anti-β-III tubulin antibody (1:500; R&D) overnight at 4°C and further stained with Alexa Fluor 488-conjugated IgG (Life Technologies) for 1 hour at room temperature. The immunofluorescence staining of corneal sections was performed with the antibodies (Table 1) according to our previous description. The quantification of corneal innervation was calculated as the percentage of area positive for β-tubulin staining as previously described. For immunofluorescence staining, frozen sections (7 μm thick) were fixed by 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 5% goat serum. The tissue samples were stained with primary antibodies, followed with fluorescein-conjugated secondary antibodies (Table 1). The staining was examined under an Eclipse TE2000-U microscope after counterstained with DAPI.

**Reverse Transcription–Quantitative Polymerase Chain Reaction**

Total RNA was extracted from control or diabetic corneal epithelium by using Nucleospin RNA Kits (BD Biosciences, Palo Alto, CA, USA). cDNAs were synthesized using the Primescript First-Strand cDNA Synthesis kit (TaKaRa, Dalian, China) according to the manufacturer’s protocol. Real time PCR was carried out using SYBR Green reagents and the Applied Biosystems 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). The cycling conditions were 10 seconds at 95°C followed by 40 two-step cycles (15 seconds at 95°C and 1 minute at 60°C). The quantification data were analyzed with the Sequence Detection System software (Applied Biosystems) using GAPDH as an internal control (Table 2).
ELISA Assay

Total protein was extracted from the mouse corneal epithelium (four eyes per group) or the cultured mouse corneal epithelial cells. The supernatants after homogenized and centrifuged (10,000g for 50 minutes at 4°C) were used for ELISA analysis according to the manufacturer’s instructions of ELISA Kit for NGF (Millipore, Bedford, MA, USA) and GDNF (DLD DEVELOP, Wuxi, China). The concentrations were calculated with the total proteins measured by using the bicinchoninic acid (BCA) kit (Beyotime, Shanghai, China).

Statistical Analysis

All the data in this study were representative of at least three independent experiments and presented as mean ± SD. Statistical analysis was performed using SPSS 17.0 software (SPSS, Chicago, IL, USA). Student’s t-test, and 1-way ANOVA. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Mouse Corneal Nerve Regeneration During Epithelial Wound Healing

To study the correlation of corneal nerve regeneration and epithelial wound healing, the central corneal epithelium was removed in normal mice. The corneal epithelial wound healing was almost completed at 2 days after the epithelial removal (Fig. 1A). Compared with the intact morphology of corneal subbasal nerve fibers in normal mice, the removal of corneal epithelium caused immediate debridement, degeneration at 24 hours (Supplementary Fig. S1), regeneration at 2 days, and partial recovery at 7 days (Figs. 1B, 1D), of the corneal subbasal nerve fibers. However, the epithelial nerve fiber terminals still remained scarce in staining at 2 days and partially recovered only at 7 days after the epithelial removal (Figs. 1C, 1E).

Neurotrophic Factor Expression During Corneal Epithelial Regeneration

To explore the expression changes of neurotrophic factors during corneal epithelial wound healing, mouse corneal epithelium including either intact, in regeneration (2 days after the epithelial removal), or fully regenerated (7 days after the epithelial removal) was collected and analyzed by quantitative RT-PCR, ELISA, and immunofluorescence staining. The results showed that among the mRNA transcripts of 12 neurotrophic factors or neuropeptides, the expression of NGF and GDNF was significantly up-regulated in the regenerating corneal epithelium, and completely or partially recovered to the basal levels in the regenerated corneal epithelium (Fig. 2A). Moreover, ELISA showed that the concentrations of NGF and GDNF were significantly increased from 27.1 ± 2.8 pg/mg and 53.4 ± 3.1 pg/mg, respectively, in the intact corneal epithelium to 50.4 ± 3.3 pg/mg and 76.9 ± 11.2 pg/mg, respectively, in the regenerating corneal epithelium, and further returned to 23.9 ± 2.6 pg/mg and 52.4 ± 7.6 pg/mg, respectively, in the regenerated corneal epithelium (Figs. 2B, 2C). Immunofluorescence staining showed that the up-regulated NGF and GDNF were mainly located in the regenerating corneal epithelium (Fig. 2D), but not in the corneal stroma. Furthermore, the protein quantification of NGF and GDNF in the conditioned medium of cultured confluent mouse corneal epithelial cells (TKE2) confirmed the up-regulated secretion after wounding (Fig. 2E). Therefore, the results suggest that corneal epithelial cells synthesize multiple neurotrophic factors, and that the secretion of NGF and GDNF are significantly up-regulated with the corneal epithelial wound healing.

NGF or GDNF Blockage on Neurite Outgrowth and Corneal Nerve Regeneration

To further demonstrate the essential role of NGF and GDNF in the promotion of corneal nerve regeneration and TG neurite

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
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outgrowth, NGF or GDNF neutralizing antibody were injected subconjunctivally during the corneal epithelial wound healing in normal mice or added in the co-culture model of TG neurons with corneal epithelial cell-conditioned medium. The injection significantly delayed the corneal epithelial wound healing (Figs. 3A, 3C) and impaired the corneal nerve regeneration (Fig. 3B) with 59% and 51% reduction of the subbasal nerve fiber density for NGF and GDNF blockage, respectively (Fig. 3D). Furthermore, the blockage of NGF or GDNF in the co-culture model also showed a significant attenuation of the TG neurite outgrowth promoted by the conditioned medium of mouse corneal epithelial cells (Figs. 3E, 3F). Therefore, the results suggest that NGF and GDNF released from the regenerating corneal epithelial cells are essential for the TG neurite outgrowth and corneal nerve regeneration.

NGF and GDNF Expression During Diabetic Mouse Corneal Epithelial Regeneration

To explore the expression changes of NGF and GDNF in diabetic mouse corneal epithelium, the intact, regenerating (2 days after the epithelial removal) and regenerated (7 days after the epithelial removal) epithelium was collected and compared with that of age-matched normal mice. The NGF and GDNF mRNA transcripts showed similar levels for diabetic and normal mice in both the intact and regenerated corneal epithelium, whereas significant differences were detected in the regenerating corneal epithelium between the diabetic and
normal mice, with lower expression in diabetic mice (Figs. 4A, 4B). The protein concentration analysis showed that the content of GDNF, but not that of NGF, was significantly decreased in the intact corneal epithelium of diabetic mice as compared to control mice. However, both the NGF and GDNF concentrations were significantly reduced in the regenerating corneal epithelium of diabetic mice when compared with that of the normal mice (Figs. 4C, 4D). Finally, neither NGF nor GDNF concentrations in the regenerated corneal epithelium showed significant differences between the diabetic and normal mice after 7 days of the epithelial removal (Figs. 4C, 4D).
Neurotrophic factors are regulatory molecules that play important roles in the growth and survival of developing neurons and the maintenance of mature neurons in both the central and peripheral nervous system. The neurotrophic factors comprise neurotrophins and axon guidance factors that are expressed by the neuron’s target tissue in order to promote the neuron survival and guide the axon growth. In skin, the neurotrophins released from keratinocytes play an important role in the neuronal maintenance, survival, and repair, while the neuropeptides released from cutaneous sensory nerves regulate the expression of neurotrophins in keratinocytes. In the cornea, previous studies have found the patterned expression of neurotrophic factors and their receptors in normal and injured conditions. In addition, our results revealed that GDNF receptor α1, the main receptor of GDNF, was expressed in mouse and human corneal epithelium (Supplementary Fig. S3). Moreover, several neurotrophic factors, such as NGF, GDNF, and ciliary neurotrophic factor (CNTF), have been reported to stimulate corneal epithelial cell proliferation, migration, and immunoprotective privilege through their receptors in corneal epithelium. Conversely, in the present study, we found that multiple neurotrophic factors were expressed in mouse intact corneal epithelium, among which NGF and GDNF were up-regulated significantly during the process of corneal epithelial and nerve regeneration. Moreover, the TG neurite outgrowth was significantly promoted by the conditioned medium of cultured corneal epithelial cells, while attenuated by the blockage of NGF or GDNF. The above findings suggest that the neurotrophic factors synthesized by corneal epithelium not only maintain corneal surface homeostasis and promote corneal epithelial wound healing, but also play an important role in promoting corneal nerve regeneration once wounded. In addition, corneal epithelium may express additional non-secreted neurotrophic factors that promote corneal nerve fiber regeneration or maintain proper nerve fiber distribution, since the direct co-culture of mouse TG neuron with cultured corneal epithelial cells (TKE2) using a microfluidic system showed more elongated and extended neurite outgrowth than that with the conditioned medium from corneal epithelial cells (data not shown). It is interesting to mention that NGF and GDNF are also the pivotal neurotrophic factors of skin cell-induced neurite outgrowth of dorsal root ganglion neurons. Moreover, keratinocytes from atopic eczema (a skin disease with higher epidermal nerve fiber density, neurogenic inflammation, and pruritus) have been shown to produce more NGF and induced an increased outgrowth of sensory nerve fibers than normal keratinocytes.

In diabetic mellitus, hyperglycemia impairs both the corneal epithelial and nerve regeneration. The changes of corneal nerve fiber density and morphology in diabetic mellitus have been considered as a window of evaluating the stage and therapeutic effects of diabetic peripheral neuropathy. Considering the main pathological corneal characteristics of neuropathy, diabetic mouse corneal epithelial wound healing provides an excellent model for the research of epithelium-nerve interactions. However, the initial factor that causes the degradation and delayed regeneration of corneal nerve fibers in diabetic mellitus remains elusive. As demonstrated in our study, the GDNF protein level in intact corneal epithelium and both the NGF and GDNF levels in regenerating corneal epithelium were significantly decreased in diabetic mice, whereas exogenous supplement of NGF or GDNF partially recovered the diabetic corneal nerve regeneration and the corneal sensitivity. The results suggest that the decreased expression of neurotrophic factors in corneal epithelium may be the cause of, or at least involved in, the nerve fiber degeneration in diabetic cornea.

In conclusion, our study demonstrates that the neurotrophic factor NGF and GDNF derived from corneal epithelium are involved in the promotion of corneal nerve regeneration, which is impaired by hyperglycemia.

**Exogenous NGF and GDNF on Diabetic Corneal Nerve and Epithelial Regeneration**

To examine if the decreased epithelial contents of NGF or GDNF contribute to the impaired corneal nerve regeneration in diabetic mice, the recombinant NGF or GDNF was subconjunctivally injected immediately after the epithelial removal in diabetic mice. Corneal subbasal nerve fiber density was measured at 2 days, and the corneal sensitivity was measured at 7 days after the epithelial removal. As shown by the whole-mount corneal nerve fiber staining, both NGF and GDNF treatment promoted faster regeneration of subbasal nerve fibers in the diabetic mice as compared to the regeneration in vehicle PBS-treated diabetic mice (Fig. 5A). Quantitative analysis revealed that NGF and GDNF treatment significantly increased the subbasal nerve fiber density and corneal sensitivity in diabetic mice (Figs. 5B, 5C), although both were still lower than in normal mice. In addition, the exogenous supplement of NGF or GDNF significantly promoted the corneal epithelial wound healing rate in diabetic mice (Supplementary Fig. S2).

**DISCUSSION**

Corneal nerve fibers exert important trophic influences on the corneal epithelium and contribute to the maintenance of a healthy ocular surface. However, exactly how the cornea maintains the proper homeostasis and nerve regeneration once injured has remained poorly understood. Previous studies of three decades ago found that the named “epithelial neurotrophic factor” secreted by rabbit corneal epithelial cells could promote the neurite outgrowth of cultured trigeminal neurons in vitro and mediate corneal nerve regeneration in vivo, although the exact factor remained unidentified.

Neurotrophic factors are regulatory molecules that play important roles in the growth and survival of developing neurons and the maintenance of mature neurons in both the central and peripheral nervous system. The neurotrophic factors comprise neurotrophins and axon guidance factors that...
Acknowledgments

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References


Figure 5. NGF and GDNF supplement on diabetic corneal nerve regeneration and sensation recovery. Central corneal epithelium was removed, and recombinant NGF or GDNF were injected subconjunctivally in diabetic mice. The overall corneal nerve fibers (A) and subbasal nerve fibers (B) were monitored at 48 hours after the epithelial debridement. The percent area occupied by subbasal nerve fibers (C) and corneal sensitivity (D) were measured and quantified (*P < 0.05).


