PURPOSE: To test if the drug FK962 (N-(1-acetylpiperidin-4-yl)-4-fluorobenzamide) facilitates axonal elongation and recovery of corneal sensitivity after creation of a corneal flap in rabbits.

METHODS: Primary cultures of rabbit trigeminal ganglion cells were used to test if FK962 promoted nerve elongation in vitro. A 130 μm-thick × 8.6 mm-diameter flap was created in rabbit corneas where topical 10^{-6} M FK962 was administered 4 times daily. After treatment of 7 days, corneal mechanical sensitivity was measured using a Cochet-Bonnet esthesiometer. Whole-mount corneal sections were prepared, sensory nerve axons were stained with antibody for neurofilament, and axonal elongation from transected nerve termini were scored using standardized criteria. Ocular pharmacokinetics modeling was used to predict permeation of topical FK962 into cornea.

RESULTS: FK962 accelerated sprouting and elongation of neurites in cultured neuronal cells from rabbit trigeminal ganglia. In the in vivo rabbit model, distal axons from transected nerve termini in corneas disappeared soon after flap surgery; but with time, axons regenerated and elongated. Topical application of 10^{-6} M FK962 for 7 days significantly enhanced axonal elongation and increased corneal sensitivity. Increased corneal sensitivity was directly and significantly correlated with axonal elongation, suggesting functional enhancement of reinnervation by FK962.

CONCLUSIONS: Results from a rabbit model of laser in situ keratomileusis (LASIK) surgery showed that topical FK962 facilitated corneal re-innervation leading to recovery of sensitivity. Results suggested that topical application of FK962 might decrease complications in patients after LASIK surgery. (Am J Ophthalmol 2012;153:651–660. © 2012 by Elsevier Inc. All rights reserved.)

LASER IN SITU KERATOMILEUSIS (LASIK) IS A WELL-ACCEPTED SURGICAL PROCEDURE FOR CORRECTING MYOPIA. HOWEVER, SENSORY NERVES IN THE SUB-BASAL (BOWMAN’S LAYER) AND SUPERFICIAL STROMAL REGIONS OF THE CORNEA ARE AMPUTATED DURING FLAP CREATION, LEADING TO NERVE DEGENERATION AND DECREASED CORNEAL SENSITIVITY. THE DENSITY OF SUB-BASAL NERVES IS SIGNIFICANTLY DECREASED 1 MONTH AFTER LASIK SURGERY, THEN GRADUALLY RETURN; BUT THE NERVES WERE STILL ABNORMAL AFTER 6 MONTHS. THESE CORNEAL NERVES ARE INVOLVED IN FEEDBACK LOOP STIMULATION OF TEAR SECRETION VIA THE SYMPATHETIC AND PARASYMPATHETIC NERVES INNERVATING THE LACRIMAL GLAND. DECREASED CORNEAL SENSITIVITY CAN THE LEAD TO COMPLICATIONS SUCH AS DRY EYE AND NEUROTROPHIC EPITHELIOPATHY.

The cornea/lacrimal gland feedback loop suggests that regeneration of corneal nerves by growth factors would help restore corneal sensitivity and be useful in therapy for dry eye. Indeed, pituitary adenylate cyclase–activating polypeptide (PACAP) induced outgrowth of neuronal processes in trigeminal ganglion cells and accelerated recovery of corneal sensitivity in a rabbit model of LASIK surgery. Nerve growth factor (NGF) has been well studied and causes neuronal outgrowth in several cell types, including trigeminal neurons. NGF is also known to be released following LASIK surgery and promotes corneal wound healing. FK962 (N-[1-acetylpiperidin-4-yl]-4-fluorobenzamide) enhanced secretion of somatostatin from rat hippocampal slices, and somatostatin induced neurite outgrowth in rat cerebellar granule cells. In cultured rat astrocytes, an analog of FK962 caused production of glial cell line–derived neurotrophic factor (GDNF), a promoter of cell survival and axonal regeneration in a wide variety of neuronal populations. The purpose of the present experiments was therefore to determine if FK962 facilitates axonal regeneration and recovery of corneal sensitivity in an in vivo model of LASIK surgery in rabbits.

METHODS

EXPERIMENTAL ANIMALS: Male Japanese white rabbits (Kitayama Labes, Nagano, Japan) at 4 days of age were the source of trigeminal ganglia for cell culture experiments, 9-
to 10-week-old male rabbits provided corneas for an in vitro trans-corneal study, and 11- to 12-week-old male rabbits were used in an in vivo model of flap creation as described in a previous report.6

• CULTURE OF TRIGEMINAL GANGLION CELLS: Trigeminal ganglia were isolated using a modification of a method previously reported.13 Minced tissues from isolated trigeminal nerves were digested for 30 minutes with 3 mg/mL collagenase A (Roche Diagnostics, Basel, Switzerland) followed by incubation for 40 minutes at 37 C in Nerve-Cell Culture System/Dissociation Solutions (Sumitomo Bakelite, Tokyo, Japan). Following filtration through a 40-μm cell strainer (Becton-Dickinson, Franklin Lakes, New Jersey, USA), cells were prepared according to the manufacturer’s protocol. Cells (1 × 10⁶) were cultured in 8-well culture dishes coated with poly-D-lysine and laminin (Becton-Dickinson) in neurobasal medium (Invitrogen/Life Technologies, Carlsbad, California, USA) supplemented with 1 mM L-glutamine (Invitrogen/Life Technologies), 0.2% primocin (Invitrogen), and 10 μM cytosine β-D-arabinofuranoside (Wako, Osaka, Japan). FK962, kindly provided by Astellas Pharma Inc (Tokyo, Japan), was used at 10⁻⁸ to 10⁻¹⁰ M.

• IMMUNOCYTOCHEMISTRY AND QUANTIFICATION OF ELONGATION IN NEURONAL CELLS: Cultured cells were fixed for 20 minutes in 2% paraformaldehyde, washed, incubated for 30 minutes in blocking solution containing 5% goat serum (Cosmo bio, Tokyo, Japan) and 0.1% Triton X-100, and incubated for 1 hour with neurofilament 200 mouse monoclonal antibody (clone N52; Sigma, St. Louis, Missouri, USA) at 1:1000 dilution. Cells were visualized by incubation for 1 hour with anti-mouse IgG conjugated to Alexa Fluor-568 (Invitrogen) at 1:1000 dilution. The digital images of cells were captured using a fluorescence microscope (IX71; Olympus, Tokyo, Japan). Quantitative assessment of neurite elongation was performed by cell counting using Image-Pro Plus ver. 4.5 (Media Cybernetics, Bethesda, Maryland, USA). A cell with an elongated neurite was defined as a cell body immunopositive for neurofilament and with a process extending to a length ≥ twice the diameter of the cell body. Neurofilament-positive cells in each well were counted under a stereoscopic microscope. Gatifloxacin antibiotic eye drops (0.3%, GATIFLO; Senju, Osaka, Japan) were administered 4 times a day for 6 days after surgery. Rabbits with initial corneal sensitivity of more than 15 mm in the right eye were selected 1 day before surgery. One day after surgery, rabbits with sensitivity less than 5 mm of filament17 were selected (n = 31). Based on corneal sensitivity before surgery, the rabbits were then divided into 2 groups using stratified randomization, resulting in mean initial sensitivities shown in Table 1. The treated group received one topical 50-μL drop containing 10⁻⁶ M FK962 in 6.4 mM phosphate-buffered saline (PBS, pH 7.0).

<p>| TABLE 1. Corneal Sensitivity After Topical Application of FK962 for 7 Days In Vivo |
|------------------------------------------|----------------|----------------|----------------|</p>
<table>
<thead>
<tr>
<th>Group</th>
<th>Day 0 (Before LASIK)</th>
<th>Day 1 (Start Treatment)</th>
<th>Day 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>(mean ± SD) 15</td>
<td>28.3 ± 6.7</td>
<td>0</td>
</tr>
<tr>
<td>FK962</td>
<td>(mean ± SD) 16</td>
<td>28.1 ± 7.3</td>
<td>0</td>
</tr>
<tr>
<td>P*</td>
<td>&gt;.1</td>
<td>.050</td>
<td></td>
</tr>
</tbody>
</table>

LASIK = laser in situ keratomileusis; SD = standard deviation. *vs vehicle (Wilcoxon rank sum test).

• RABBIT MODEL OF FLAP SURGERY: A corneal flap was created in the right eye of anesthetized rabbits from the nasal side at the center of the cornea using an automated corneal shaper microkeratome (Nidek, Aichi, Japan) with a planning thickness of 130 μm and a diameter of 8.6 mm.6 The corneal flap was returned in place without sutures under a stereoscopic microscope. Gatifloxacin antibiotic eye drops (0.3%, GATIFLO; Senju, Osaka, Japan) were administered 4 times a day for 6 days after surgery. Rabbits with initial corneal sensitivity of more than 15 mm in the right eye were selected 1 day before surgery. One day after surgery, rabbits with sensitivity less than 5 mm of filament17 were selected (n = 31). Based on corneal sensitivity before surgery, the rabbits were then divided into 2 groups using stratified randomization, resulting in mean initial sensitivities shown in Table 1. The treated group received one topical 50-μL drop containing 10⁻⁶ M FK962 in 6.4 mM phosphate-buffered saline (PBS, pH 7.0).
in the right eye 4 times a day for 7 days, while the vehicle group received PBS.

**CORNEAL SENSITIVITY MEASUREMENTS:** Corneal sensitivity was measured at the center of the cornea with a Cochet-Bonnet esthesiometer (Luneau, Paris, France) under a double-masked procedure 1 day before and 1 and 8 days after surgery. The diameter of the nylon filament was 0.12 mm, and the length could be varied from 60 mm (maximum sensitivity) to 5 mm (minimum); lower than 5 mm was recorded as 0 sensitivity.

Since use of the esthesiometer is limited because rabbits are poor blinkers and generally not cooperative, the following procedures were used to eliminate false positives attributable to excited animals blinking simply because of an approaching instrument. 1) Each animal was gently held in a custom-made restraint box and placed in a quiet room (noise of 30-40 dB) during measurement. 2) If an excited animal moved his head during measurement, the measurements were restarted after sedation. 3) Rabbits with half-closed eyes were eliminated. 4) The upper and lower cilia were trimmed off, and hair debris on the corneal surface and nictitating membrane were gently removed using forceps. 5) A positive response was recorded if the eyelid reflex closed immediately after touching the nylon filament perpendicularly to the center of the corneal surface and if ≥5 reflexes occurred in 10 consecutive touches. Each sensitivity test was repeated 3 times, advancing in 5-mm steps. Two positive responses in the 3 attempts at each filament length were regarded as a positive result. The longest filament length causing a positive result was considered the threshold of sensitivity for that cornea. These procedures produced reliable data, but were so labor-intensive that experiments were limited to 7 days.

**IMMUNOHISTOCHEMICAL STAINING OF CORNEAL NERVES:** Corneoscleral buttons were obtained from globes after treatment with FK962 or vehicle for 7 days. To allow penetration of antibodies into cornea, previous methods were modified. Corneal endothelium was scraped off, and the button was immersed in ice-cold saline containing 5 mM EDTA for 1 hour to hydrate the stromal layer. The button was then fixed overnight in Zamboni's solution containing 4% paraformaldehyde and 0.1% picric acid in 0.1 M phosphate buffer, pH 7.4. The button received four 30-minute rinses in 70% ethanol at 4 C, followed by incubation overnight at −30 C in dimethyl sulfoxide (DMSO)/methanol (1:4). After rinsing 2 times for 30 minutes each with 100% methanol, the button was subjected to 4 cycles of freezing and thawing between −80 C and room temperature to break up the plasma membrane. The button was rehydrated in 70%, 50%, and

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**FIGURE 1.** Scores for regeneration of corneal nerves in the present rabbit model of flap surgery. (Top row) Scores with diagrams describing nerve regeneration. (Middle row and Bottom row) Immunofluorescence confocal microscopy for neurofilament in flat-mounted corneas in (Middle row) vehicle-treated and (Bottom row) FK962-treated rabbits, showing stromal nerve trunks (white arrowheads) and subepithelial nerve plexuses (red arrowheads). After flap creation (gray area), transected nerve termini were observed in the corneal bed (black and white arrows). During healing, the transected nerves elongated in horizontal (green arrowheads) and vertical (blue arrowheads) directions. Each photograph shows the maximum-intensity image along the z-axis in a stack of x-y images. Horizontal bar is 100 μm.
15% methanol and phosphate buffer for 30 minutes each. The sclera was removed from the button and 4 radial incisions were made in the remaining cornea. Incised tissues were immersed for 30 minutes in phosphate buffer containing 30% sucrose, followed by OCT compound (Sakura Finetech, Tokyo, Japan) for 3 minutes and flat-mounting by freezing. The Descemet membrane was removed with a freezing microtome, and the corneal tissue was thawed in PBS.

The cornea was free-rotated overnight at 4°C in PBS blocking solution containing 2% skim milk, 5% DMSO, and 0.1% Triton X-100 and then incubated for 2 days with primary monoclonal antibody for neurofilament 200 at 1:1000 dilution. After three 90-minute washes in blocking solution, the tissue was incubated overnight with goat anti-mouse IgG conjugated to Alexa Fluor 488 or 568 (Invitrogen) at 1:200 dilution. After three 90-minute washes in PBS containing 5% DMSO, the tissue was immersed in Vectashield (Vector, Burlingame, California, USA) and flat-mounted with the epithelium side down on glass-bottom culture dishes. Images of nerve axons were observed using a laser scanning confocal microscope (LSM710; Carl Zeiss, Hallbergmoos, Germany) and digitized with ZEN 2000 software. Axonal regeneration was assessed in a 2-mm-diameter circular area at the center of the cornea, where corneal sensitivity had been measured, and was scored using defined criteria as follows (Figure 1): score 0, no nerve fibers or only transected nerve termini; score I, horizontal axonal elongation along the flap-bed interface (green arrowheads); score II, horizontal and vertical axonal elongation into the flap (blue arrowheads).

Normal corneas were also stained for nerves and Schwann cells. Anti–substance P (SP) primary monoclonal antibody (1:200 dilution, clone NC1; Chemicon, Temecula, California, USA) and goat anti-rat IgG conjugated to Alexa Fluor 568 (1:200 dilution, Invitrogen) were used to visualize Aδ and C fibers. Primary monoclonal antibody conjugated to Cy3 (1:200 dilution, clone G-A-5; Sigma) without Zamboni’s fixative stained for glial fibrillary acidic protein (GFAP) in Schwann cells. Washing buffer containing 5 μg/mL DAPI (Dojindo, Tokyo, Japan) was then used to stain for nuclei before flat-mounting the immunostained tissues.

**STATISTICAL ANALYSIS:** Statistical analyses were performed using software (SAS ver. 9.1 or JMP ver. 8.0; SAS Institute, Cary, North Carolina, USA). The normality of the data distribution was tested using the Shapiro-Wilk test, with the cut-off for non-normality set at \( P < 0.05 \), and then in the case of normal distribution, the Dunnett test was performed for analyzing cell numbers/neurite density.
elongation. Corneal sensitivity and nerve morphologic scoring were analyzed using a 1-tailed nonparametric Wilcoxon rank sum test. Spearman rank correlation coefficient was used to determine direct correlation between morphologic scores and corneal sensitivity.

RESULTS

● SENSORY NERVE FIBERS IN RABBIT CORNEA: Trigeminal ganglion contains sensory neurons classified as myelinated Aα, Aβ, and Aδ fibers; and as unmyelinated C fibers. The innervation of cornea in rabbits by Aδ and C neurons has been demonstrated by electrophysiology, but not by immunohistochemistry, and the fibers originate from the ophthalmic division of the trigeminal ganglion. Further, almost all of the A-type neurons were found to stain by anti-NF200 antibody and some Aδ and C neurons were stained by anti-SP antibody located in the dorsal root ganglion. To assess axonal elongation after flap surgery in our rabbit study, we therefore first established the immunohistochemical staining for corneal Aδ and C fibers in normal cornea. We found that nerve fibers in normal corneas from rabbits showed 3 staining patterns: NF200-positive/SP-negative Aδ fibers (green, after image merging, Figure 2, Top); NF200-positive/SP-positive, possible Aδ fibers (yellow after merging); and NF200-negative/SP-positive fibers, possible C fibers (red after merging). NF200-positive nerves were also found to be ensheathed by myelinating GFAP-positive Schwann cells (red, Figure 2, Bottom). These data suggested that NF200 was the better marker for detection of Aδ fibers, which are believed to be a main mechano-sensory fiber in cornea.

● FK962 ENHANCED NEURITE ELONGATION IN VITRO: Primary rabbit trigeminal ganglion cells staining positive for neurofilament (red) were round and without sprouting after 10 hours of culture, in both control (Figure 3, Top, control row) and FK962-treated groups (Figure 3, Top, 10^{-10} M FK962 row). At 12 hours, control cells started sprouting neurites (Figure 3, Top, control row, arrowhead), and treatment with FK962 enhanced neurite elongation.

FIGURE 3. Effect of FK962 on neurite elongation. (Top) Representative immunofluorescence microscopy of cultured rabbit trigeminal ganglion cells stained red for neurofilaments, showing neurite sprouting (arrowhead) and subsequent elongation (arrows). (Bottom) Cell counting showing that FK962 at 10^{-10} and 10^{-9} M significantly increased the number of cells with neurite outgrowth. Data are means ± SD (n = 23). *P < .05 vs control (Dunnett test).
At 17 hours, the number of cells with elongated neurites was further increased by FK962. Cell counting produced a bell-shaped dose response (Figure 3, Bottom), showing that FK962 at $10^{-10}$ and $10^{-9}$M significantly increased the percentage of cells with elongated neurites compared to controls. The higher dose at $10^{-8}$M did not enhance elongation. These histologic observations and cell quantification data established that FK962 stimulated neurite elongation in vitro. They also suggested that effective dosing levels and protocols used for in vivo treatment would need to cause accumulation of $10^{-10}$ to $10^{-9}$M levels of FK962 within the intact corneal layers.

**IN SILICO UPTAKE OF TOPICAL FK962 INTO CORNEAS WITH FLAP SURGERY**: FK962 permeated through corneas across the aperture of an Ussing chamber, and FK962 accumulated with increasing incubation time (Figure 4, Top left). Corneas without epithelium (open squares) allowed more accumulation as compared to side-by-side mounted, intact corneas (closed squares), indicating that epithelium was a permeability barrier to FK962. These plots were used to determine lag times and the permeation rates of FK962 into cornea. Calculated permeation rate and lag time in intact cornea with flap creation were $65.9 \pm 5.6 \mu g/cm^2/hour$ and $0.20 \pm 0.09$ hours, respectively. In de-epithelialized cornea, permeation rate increased to $157.7 \pm 47.3 \mu g/cm^2/hour$ and lag time decreased to $0.06 \pm 0.03$ hours.

TABLE 2. Parameters Used for Pharmacokinetics Modeling of Topical FK962 in Corneas From Rabbits With Flap Creation

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diffusion coefficient (epithelium) (cm$^2$/sec)</td>
<td>$1.56 \times 10^{-8}$</td>
</tr>
<tr>
<td>Diffusion coefficient (stroma) (cm$^2$/sec)</td>
<td>$9.74 \times 10^{-7}$</td>
</tr>
<tr>
<td>Partition coefficient (stroma/buffer) (-)</td>
<td>7.05</td>
</tr>
<tr>
<td>Partition coefficient (epithelium/buffer) (-)</td>
<td>1.64</td>
</tr>
<tr>
<td>Total corneal thickness (cm)</td>
<td>0.0400</td>
</tr>
<tr>
<td>Epithelium thickness (cm)</td>
<td>0.0035</td>
</tr>
</tbody>
</table>
minutes after administration, FK962 dosing at 3
levels (Figure 4, Top right, C lines). Except for 2
lium and stroma to the endothelium (Figure 4, Bottom
produced a fairly constant distribution across the epithe-
10
hours (Figure 4, Top right, B lines); virtual instillation at
application, peak concentrations of FK962 in the epithe-
modeling of drug concentrations in corneal layers after
different dosing regimes. After a simulated 1-time topical
application, peak concentrations of FK962 in the epithe-
ium occurred at 1.3 minutes after application and at 10
minutes in the stroma, and then gradually decreased with
similar profiles for 3 different dosages (Figure 4, Top right).
Note that 3 × 10⁻⁶ M FK962 resulted in 10⁻⁹ and 10⁻¹⁰
M FK962 in the stroma (effective concentration in cul-
ted neurons; Figure 3) persisting for approximately 2
hours (Figure 4, Top right, B lines); virtual instillation at
10⁻⁶ M FK962 persisted for 3 to 5 hours (Figure 4, Top
right, A lines); but 10⁻⁹ M FK962 produced sub-effective
corneal levels (Figure 4, Top right, C lines). Except for 2
minutes after administration, FK962 dosing at 3 × 10⁻⁸ M
produced a fairly constant distribution across the epithe-
lium and stroma to the endothelium (Figure 4, Bottom
left), where FK962 would be needed for axonal elongation
in vivo. Simulated topical application of FK962 4 times at
2-hour intervals caused persistence of effective levels of
FK962 in corneal stroma concentrations for 8 hours
(Figure 4, Bottom right). These in silico studies suggested
that multiple dosing of topical FK962 was necessary to
produce effective and sustained levels of FK962 in corneal
stoma. Preliminary in vivo tests with 4× multiple dosing
showed that 10⁻⁶ M FK962 (but not 3 × 10⁻⁸ M) tended
to accelerate recovery of corneal sensitivity after flap
surgery and that corneal sensitivity normally decreased to
its lowest level within 1 week after flap surgery and then
recovered at a slow, constant rate (data not shown). Taken
together, all these data justified the in vivo protocol below
using multiple dosing at 10⁻⁶ M topical FK962 and
assessment of FK962 efficacy for flap recovery during the
first week after flap surgery.

**Efficacy of FK962 in Flap Recovery:** After flap
surgery in our in vivo rabbit model, distal axons of
transected corneal nerve termini completely disappeared
(Figure 1, score 0), and new axons soon regenerates and
elongated (Figure 1, scores I and II). This occurred in both
vehicle-control and FK962-treated animals, and the mi-
croanatomy of FK962-regenerated axons appeared normal
(Figure 1, Vehicle and FK962 rows). Moreover, topical
application of 10⁻⁶ M FK962 for 7 days significantly
accelerated elongation of transected nerves, as evidenced
by the increased frequency of more innervated score II
axons in the FK962 group (Figure 5, Top). Corneal
sensitivity fell to 0 mm 1 day after flap surgery, and topical
application of 10⁻⁶ M FK962 for 7 days significantly
accelerated an increase in sensitivity (Table 1). Impor-
tantly, corneal sensitivity was found to be significantly and
directly correlated with elongation of axons caused by
FK962 (Figure 5, Bottom), suggesting that Aδ may be
major fibers for mechanistic sensitivity.

**Discussion**

The major finding of the present study was that
topical FK962 promoted axonal elongation in vivo, which
accelerated recovery of corneal sensitivity. This conclusion
was supported by findings that FK962 promoted neurite
elongation in cultured cells (Figure 3), rapidly reached
constant and effective tissues levels in flapped cornea
modeling studies (Figure 4), and accelerated axonal elon-
gation (Figures 1 and 5) and corneal sensitivity (Table 1)
in a rabbit model of corneal flap regeneration.

**Mechanism of Action of FK962 in Vitro:** Cul-
tured trigeminal ganglion cells contain a mixture of glial
cells such as Schwann cells and sensory neurons such as
Aαβ, Aδ, and C, along with other cell types. Since Aδ
cells are twice as abundant as Aαβ cells, this sug-

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**Figure 5.** Score for axonal elongation and correlation with
sensitivity in cornea after topical application of FK962 for 7
days in vivo. (Top) Significant acceleration by FK962 in axonal
elongation. n = 15 for vehicle and n = 16 for FK962. *P = .046 vs vehicle (Wilcoxon rank sum test). (Bottom) Corneal
sensitivity in individual eyes from vehicle-treated (open dia-
monds) and FK962-treated (closed diamonds) rabbits. The eyes
were grouped on the x-axis according to the corneal morphology
scores (axonal elongation) as defined in Figure 1. When using
all data, a statistically significant, positive correlation between
corneal sensitivity and morphology scores was observed (Spear-
man rank correlation coefficient ρ = 0.7). P < .0001 (n = 31).

These calculated values were allowed calculation of diffu-
sion and partition coefficients (Table 2) necessary for
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cells are twice as abundant as Aαβ cells, this sug-
gested that our culture cells could be related to the situation in intact cornea. In the cultured cells, FK962 was not effective at higher concentrations (Figure 3, Bottom), and, likewise, FK962 exhibited a bell-shaped dose response in hippocampal slices.9 Many neurotrophic factors and peptides causing axonal elongation show bell-shaped dose responses, 31 related to negative feedback mechanisms.32,33 FK962 caused release of somatostatin in brain tissues, 9 and an analog FK960 caused release of GDNF, 11 suggesting that the mechanism of action of FK962 causing neurite elongation in culture may be through stimulating the release of somatostatin and/or GDNF from cultured cells.

- MECHANISM OF ACTION OF FK962 IN VIVO: Topical application of FK962 in our rabbit model accelerated axonal elongation after flap surgery. Although the antibody for neurofilament would stain both axons of Aβ and Aδ fibers, only Aδ is present in the cornea.25 Topical application of neurotrophic factors and peptides that induced axonal elongation also accelerated recovery of corneal sensitivity.6,10 Axonal elongation was directly related to corneal sensitivity, suggesting a cause/effect mechanism that may become clinically useful in the future.

The functions of corneal sensory nerves are heterogeneous, for example, mechanical and thermal Aδ fibers, polymodal C fibers, and cold-sensitive Aβ and C fibers.29 These fibers extend from the trigeminal ganglion and terminate in free nerve endings.34 The Cochet-Bonnet esthesiometer used in the present experiment detects mechanical excitation,35 and measured sensitivity was well correlated with the elongation scores for Aβ fibers, suggesting reception of mechanical stimuli by Aβ fibers in our rabbit model. Aβ fibers in corneal stroma are surrounded by Schwann cells, which may be the source of neurotrophic factors such as GDNF. Thus, as in vitro, FK962-induced release of somatostatin and/or GDNF may be the mechanism for axonal elongation and recovery of mechanical sensitivity after flap surgery.

One limitation of the present experiments was that assessment of axonal regeneration of C fibers was not performed. Although Aδ fibers are likely to be the major mechano-sensory fibers,26 decreased mechanical sensitivity induced by capsaicin was mainly associated with damage of unmyelinated fibers,36 suggesting involvement of C fibers. Thus, it will be worthwhile to determine if FK962 also induces axonal elongation in C fibers.

Another limitation of the present study was that we tested the effect of FK962 at early stages of recovery (7 days) and found recovery of corneal sensitivity was significantly accelerated by topical application of FK962. Recovery was faster in our rabbit model than in humans, where corneal sensitivity was reported to be lowest at approximately 7 days after LASIK surgery; and when complications occur, such as a decrease in tear secretion, they are usually at 2 weeks after LASIK surgery.37,38 In humans, preservation of corneal sensitivity after flap creation led to better recovery of corneal sensitivity and suppression of concomitant complications 3 months after surgery.10 We expect persistence of the FK962 effects into the later stages of corneal recovery, but this needs to be verified with long-term observations.

Pharmacokinetics modeling in corneas could provide useful predictions as to how topical application of drugs may be transported and accumulated by cornea after LASIK surgery. Topical FK962 rapidly penetrated the cornea, where the epithelium acted as a permeability barrier. After the initial application, the bulk of the epithelium and stroma reached nearly equal concentrations of FK962. Note that the actual dose (10⁻⁸ M) of FK962 required for axonal elongation and recovery of sensitivity after flap surgery was higher than predicted by the pharmacokinetics model (3 × 10⁻⁸ M). Reasons for this difference include: duration of exposure was continuous for 17 hours in vitro while only approximately 8 hours/day in vivo, cell populations were different in the in vitro (Aα/β and Aδ cells) and the in vivo (only Aδ) models, and inflammation from flap surgery may have altered the corneal barrier regulating delivery of FK962.40

In summary, FK962 promoted nerve regeneration and accelerated recovery of corneal sensitivity after flap creation in a short-term (7-day) live rabbit model. Recovery of corneal sensitivity at the early stages of recovery may be important for preventing diseases attributable to dry eye. Our results suggested that FK962 might be a good candidate drug for prevention or improvement of complications after LASIK surgery.
REFERENCES


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