Corneal Nerve Damage and Regeneration After Excimer Laser Photokeratectomy in Rabbit Eyes

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Purpose. To investigate corneal nerve damage and its subsequent regeneration after excimer laser photokeratectomy in rabbit eyes.

Methods. Corneal photokeratectomy was performed on the right eyes of 34 pigmented rabbits, while the left eyes were used as a control. A Summit UV 200 excimer laser (Summit Technology, Waltham, MA) was used to produce a 100 μm depth phototherapeutic keratectomy (4.5 mm diameter). The rabbits were killed at 0, 1, 2, 3, 4, 5, 7, 8, 10, 15, and 20 days and 1, 2, 3, and 4 months after treatment. Corneal nerve regeneration was investigated using a monoclonal antibody directed against a 150 kD subunit of neurofilaments, in combination with acetylcholinesterase staining.

Results. At time 0, the subepithelial plexus had disappeared completely from the photoablated area. One day after treatment, some regenerating nerves sprouting within the treated area were visualized by a monoclonal antibody directed against a 150 kD subunit of neurofilaments. Acetylcholinesterase staining was detectable 7 days later. One month after surgery the subepithelial plexus had already reappeared, but was morphologically disorganized. Four months after treatment, the newly formed subepithelial plexus had considerably thickened in comparison with that of the control eye.

Conclusions. This study shows that corneal nerve regeneration after excimer laser photorefractive keratectomy in rabbit eyes, although more intense, is similar to that produced after other surgical procedures involving the epithelium and the anterior stroma or one of the two. Invest Ophthalmol Vis Sci. 1994;35:229–235

It has been demonstrated that 193 nm excimer laser photoablation can produce an accurate excision of corneal stroma with minimal damage to the remaining tissues.1,2 Excimer laser removal of superficial stroma can be used to perform central lamellar keratectomy to modify the refractive power of the cornea.3,4 Excimer laser phototherapeutic keratectomy (PTK) has also been shown to be effective in the treatment of patients with superficial corneal opacities.5 Furthermore, the results of some recently published multicenter clinical studies have confirmed that excimer laser photorefractive keratectomy is safe and effective in reducing low and mild myopia.6,7 However, on the day after laser sessions, patients have had troublesome ocular pain requiring analgesic medical therapy, and a measurable decrease in corneal sensitivity persisting 3 to 4 months has also been reported.8 Normal corneal innervation involves both the basal epithelial cell layer and a plexus located immediately beneath the epithelium in the anterior stroma.9 Given that the excision depth may vary from 10 to 100 μm (depending on the attempted correction), it is presumed that photokeratectomy removes both the epithelial and subepithelial nerve plexus. However, although corneal hypesthesia and anesthesia are probably due to the nerve damage produced by laser photoablation, precise knowledge of the morphology of such damage and its subsequent regenerative patterns is still lacking.

The aim of this study was to evaluate the morphology of corneal nerve regeneration after excimer laser photoablation in rabbit eyes.

MATERIALS AND METHODS

Laser System

The laser system used was a Summit UV 200 (Summit Technology, Waltham, MA). The operative energy...
fluence and repetition rate were set at 180 mJ/cm² and 10 Hz, respectively, and the cut rate at 0.25 μm per pulse.

**Laser Treatment**
The animals used in this study were treated in accordance with the ARVO Resolution on the Use of Animals in Research.

Thirty-four pigmented adult rabbits (2.5 to 3 kg) received pretreatment systemic sedation by means of an intramuscular injection of ketamine 25 mg/kg. Oxybuprocainium chlorathum (0.4%) was used for topical anesthesia. Excimer laser PTK was performed on the right eye, with the left being used as a control. The depth of ablation was 100 μm (pulse number 400) with a diameter of 4.5 mm; the corneal epithelium was directly removed by means of the excimer laser beam.

**Morphologic Study**
The animals were killed using an overdose of intravenous sodium pentobarbital at 0, 1, 2, 3, 4, 5, 7, 8, 10, 15, and 20 days, and 1, 2, 3, and 4 months after the treatment. Before death, the animals underwent biologic examination of the anterior segment of the eye.

The corneas were removed after enucleation and, after a brief rinse with phosphate-buffered saline (phosphate buffer 20 mM, pH 7.4, 0.9% NaCl), were fixed with formaldehyde 4% (freshly prepared from paraformaldehyde) in phosphate-buffered saline for 2 to 3 hours at 4°C. After another phosphate-buffered saline rinse, the tissues were infiltrated with 15 to 18% sucrose and the inferior quadrants stained for the histological detection of acetylcholinesterase (AchE).

**Immunostaining.** To allow penetration of the immunoreagents into the corneal tissue, the superior quadrants were frozen in liquid nitrogen and 20 μm thick longitudinal serial sections were cut at 200 μm intervals. For each cornea, 30 sections were collected. Immunolabeling was carried out using a monoclonal antibody directed against the neurofilament 150 kD subunit (donated by M. Vitadello, MD), followed by fluorescein isothiocyanate goat anti-mouse immunoglobulins.

A solution containing phosphate-buffered saline pH 7.3, 1% bovine serum albumin and 0.3% Triton X-100 was used to dilute the immunoreagents and to rinse the sections between incubations.

**AchE staining.** AchE staining was performed using Karnovsky’s technique: the cornea specimen were incubated for 30 minutes at room temperature in 10 ml of solution containing 65 mM Na acetate buffer pH 5, 10 mM Na citrate, 3 mM CuSO4, and 0.5 mM FeCNK; the samples were then transferred to 10 ml of the same solution containing 10 mg of acetyltyocholine. Incubation was carried out at 4°C for 10 to 15 hours. Specificity control consisted in incubating the samples in the reaction solution without acetyltyocholine.

All of the micrographs were taken using a Zeiss Axiophot photomicroscope (Oberkochen, Germany).

**RESULTS**
After photoablation, the epithelium covered the treated area within 2 ± 1 days (mean ± SD).

**Immunostaining.** Figure 1 shows immunolocalization of a monoclonal antibody directed against the neurofilament 150 kD subunit in a normal eye, with a subepithelial nerve entering the epithelium (A) and a deep stromal nerve trunk (B).

A few hours after PTK, immunoreactivity of a monoclonal antibody directed against the neurofilament 150 kD subunit was detectable in the stroma beneath the treated area. This can be attributed to the presence of large nerve trunks located deep within the stroma that appeared to be unaffected by PTK.

One day after treatment, thin nerve branches intensely stained by a monoclonal antibody directed against the neurofilament 150 kD subunit were frequently seen within the stroma close to the regenerating epithelium. These branches were seen both on the periphery and in the central portion of the ablated area. In this latter location, they were difficult to distinguish from the deep unaffected trunks. Figure 2 shows one of these branches photographed at the distance of 700 μm from the periphery of the ablation.

During subsequent days, the density of the new growing branches steadily increased. By 1 month, the invasion of the stroma was almost complete and later, the subepithelial plexus was thicker than in controls and the deep stromal trunks usually had a tortuous path.

**AchE staining.** Whole-mount observation of AchE-stained corneas confirmed that, at time 0, the ablated area was devoid of the subepithelial nerve plexus, while most of the deep stromal trunks appeared to be unaffected (Fig. 3). The periphery of the lesion showed the abrupt interruption of the subepithelial nerves at the periphery of the lesioned area (Fig. 3).

Seven (± 2) days later, AchE staining revealed fine fibers randomly sprouting from the deep stromal trunks beneath the lesion and from the peripheral subepithelial plexus (Fig. 4, A and B). Frequently, fine regenerating axons were seen projecting anteriorly to the epithelium.

In subsequent days, the number and tortuousness of the regenerating axons increased both at the periphery (Fig. 5A) and at the center of the lesioned areas (Fig. 5B).
FIGURE 1. Immunofluorescence of a monoclonal antibody directed against the neurofilament 150 kD of normal rabbit cornea. The white arrow shows the subepithelial nerve plexus entering the basal epithelial cell layers (A) and a deep stromal nerve trunk (B) (400 X).

FIGURE 2. Immunofluorescence of a monoclonal antibody directed against the neurofilament 150 kD of the lesioned area 1 day after treatment. Beneath the epithelium (white arrows), regenerative nerve sprouting is evident (400 X).

FIGURE 3. Border of the lesion (acetylcholinesterase staining) at time 0: the subepithelial plexus appears to be interrupted (small black arrows); the larger arrow shows the unaffected deep stromal trunk (200x).
FIGURE 4. (A) Border of the lesion 1 week after treatment (acetylcholinesterase staining). The large black arrow shows the initial sprouting of fine fibers from the deep stromal nerves. Note that the sprouting (small black arrows) involves not only unaffected deep stromal trunks but also their superficial lesioned branches (200 X). (B) Center of the lesion 7 days after treatment (acetylcholinesterase staining). Similar sprouting (black arrows) characterizes the central area of the lesion (200 X).

This process continued steadily and 1 month after PTK, neurite density in the subepithelium of the treated eyes was higher than in controls, with a network of fine neurites filling the center of the lesion (Fig. 6). At this time, a few axonal leashes were seen intermingled with the epithelial cells.

Three to four months after treatment the subepithelium plexus was still thicker (Fig. 7A) than that of the control eye (Fig. 7B), while the morphology and density of the intraepithelial leashes were similar (Fig. 8, A and B).

DISCUSSION

Corneal nerve regeneration after mechanical, physical, and chemical lesions has been investigated in both animals and humans, but the recently introduced technique of excimer laser photoablation causes a new type of corneal nerve damage. In 1990, Pallikaris et al. described a comparative study of neuronal regeneration after corneal wounds induced in rabbit eyes by means of an argon fluoride excimer laser and mechanical methods. Using the gold chloride impregnation technique, they demonstrated that the morphology and time course of nerve fiber regeneration were similar after both mechanical and excimer laser surgery. However, no information was given concerning the time lapse between nerve sprouting and AchE detection. Furthermore, because the corneas were examined for up to 4 weeks after laser lamellar keratectomy, long-term morphologic modifications of the plexi were not assessed.

The aim of this study was to evaluate the morphologic and biochemical changes in the corneal nerve
after deep laser keratectomy involving the removal of both the epithelial and subepithelial plexus. To achieve laser ablation of the corneal epithelium and anterior stroma, together with the subepithelial nerve plexus, 400 pulses were required (target ablation depth: 100 μm). PTK was used instead of photorefractive keratectomy to ensure a well-defined lesion of the subepithelial nerve plexus with a regular shape and borders. AchE staining was used to investigate the pattern of neural healing; immunofluorescence was used to obtain more detailed information concerning the beginning of the neurofilament process. It has been shown that the appearance of neurofilaments in the distal segment of damaged neurites represents the initial phase of neural regrowth. 10,12

Shortly after laser keratectomy, AchE staining showed a complete loss of the subepithelial plexus within the treated area, whereas the large nerve trunks located deeply in the stroma appeared to be unaffected (Fig. 3). In the surrounding area, the nerves appeared to be normal and truncated at the margin of the lesion (Fig. 3). No necrosis was observed in the stroma beneath the ablation surface. Neurofilament immunoreactivity was detected in the ablated stroma as early as 24 hours after treatment (Fig. 2), suggesting that nerve regeneration is more immediate after PTK than after other types of corneal damage. 13,14

FIGURE 7. Four months after treatment (acetylcholinesterase staining): (A) the neoformed subepithelial plexus is considerably thicker than that of the control eye (B) (400 X).
Histologic examination of excimer laser photoablation has shown that tissue damage is confined to electron-dense “pseudomembrane” material (0.05 to 0.10 µm thick), and that no damage is caused to underlying tissues. Free from necrotic cellular debris, the new smooth surface covered by the pseudomembrane seems to act as a substitute for Bowman’s membrane, allowing regular and early reepithelialization. There could be a relationship between the rapid onset of nerve regeneration observed in the stroma and the immediate proliferation of the epithelium covering all the ablated area. A relationship between the rate of functional axonal regeneration and the presence of epithelial or other types of cells releasing neurotrophic factor(s) has been reported previously. Further studies should be done to clarify the role of the epithelium in the stromal nerve regeneration.

The observed gap of about 7 days between stromal nerve sprouting and AChE detection (Fig. 4, A and B) has also been reported after mechanical wounding of the cornea. This finding seems to be associated with a normal nerve recovery process in which it takes several days before AChE can be detected in the new fibers.

In our study, nerve healing reaction was quite intense. Regeneration was observed starting from both the interrupted and the unaffected deep stromal nerves. Four months after laser treatment, the density of neurites in the subepithelial plexus was higher than that in the control eyes (Fig. 7, A and B). A similar finding has been reported by Chan-Ling et al after surgical keratotomy in the cat. Several factors seem to influence corneal neural regrowth, including the type and extent of the corneal damage and the metabolic condition of the cornea. In his experimental study on rabbits, Goodman showed that there is a critical ablation depth beyond which scarring may occur and that a 100 µm deep excision produces stroma opacification indicating fibrous deposition. In our study, biomicroscopic evaluations performed during the follow-up period revealed a slight stromal haze, which was still present 4 months after treatment. Some studies have shown that various extracellular matrix proteins (such as collagens, fibronectin, laminin, and certain glycosaminoglycans) promote neurite extension. Therefore, it is possible that the depth of the ablation used in our study might have played an important role in stimulating stromal healing by intensifying the production of nerve stimulating substances, although further studies are necessary to evaluate the possible relationship between corneal nerve regeneration and collagen regrowth or epithelial hyperplasia.

In conclusion, the results of this study show that corneal nerve regeneration after excimer photokeratectomy in rabbit eyes, although more intense, is similar to that produced after other surgical procedures involving the epithelium and/or the anterior stroma.

Key Words
corneal nerve, excimer laser, photokeratectomy, rabbit

References
Corneal Nerve Regeneration and Laser Photokeratectomy


