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Degeneration and Regeneration of Subbasal Corneal Nerves after Infectious Keratitis

A Longitudinal In Vivo Confocal Microscopy Study

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Purpose: To investigate the longitudinal alterations of subbasal corneal nerves in patients with infectious keratitis (IK) during the acute phase, cessation of treatment, and the recovery phase by in vivo confocal microscopy (IVCM).

Design: Prospective, longitudinal, case-control, single-center study.

Participants: Fifty-six eyes of 56 patients with the diagnosis of bacterial (n = 28), fungal (n = 15), or *Acanthamoeba* (n = 13) keratitis were included in the study. Thirty eyes of 30 normal volunteers constituted the control group.

Methods: Corneal sensation and serial IVCM of the central cornea were performed prospectively using the Heidelberg Retina Tomograph 3/Rostock Cornea Module (Heidelberg Engineering, Heidelberg, Germany). The IVCM images were assessed at 3 time points: at the acute phase (first visit to the cornea service), at cessation of antimicrobial treatment, and up to 6 months after the resolution of infection.

Main Outcome Measures: Total nerve number and length, main nerve trunks, branching, and corneal sensation were assessed during the follow-up period.

Results: Corneal nerves were reduced significantly during the acute phase in eyes with IK compared with controls across all subgroups, with total nerve length of 5.47±0.69 mm/mm^2 versus 20.59±1.06 mm/mm^2 (P <0.0001). At the cessation of treatment, corneal nerves in patients with IK had regenerated, including total nerve length (8.49±0.94 mm/mm^2; P = 0.02) and nerve branch length (4.80±0.37 mm/mm^2; P = 0.005). During the recovery phase, after resolution of infection, corneal nerves regenerated further, including total nerve length (12.13±1.97 mm/mm^2; P = 0.005), main nerve trunk length (5.80±1.00 mm/mm^2; P = 0.01), and nerve branch length (6.33±0.76 mm/mm^2; P = 0.003) as compared with the acute phase, but were still significantly lower when compared with controls (P < 0.05 for all parameters). Corneal degeneration and regeneration correlated with corneal sensation (r = 0.47; P = 0.0009).

Conclusions: Patients with IK who sustain profound loss of corneal nerves during the acute phase of infection demonstrate increased corneal nerve density during the first 6 months after the resolution of infection. However, despite significant nerve regeneration, corneal nerve density does not recover fully and remains low compared to controls. By providing an objective methodology to monitor corneal re-innervation, IVCM adds potentially important findings that may have implications for clinical management and surgical planning. *Ophthalmology 2015;■:1–10 © 2015 by the American Academy of Ophthalmology.*

Infectious keratitis (IK) is a vision-threatening disease that varies in incidence depending on geographic location and predisposing risk factors. It is estimated that 30 000 new cases of IK (including bacterial, fungal, and *Acanthamoeba*-associated disease) occur annually in the United States.[1,2] These micro-organisms trigger an immune response, leading to severe corneal inflammation, ulceration, and scarring.[1,3–8] Corneal ulceration and subsequent scarring may result in corneal nerve damage with clinical consequences due to impaired corneal nerve function.

The human cornea is supplied by the terminal branches of the ophthalmic division of the trigeminal nerve and is the most densely innervated tissue of the body.[1,4,9–14] Nerves penetrate the cornea in the deep peripheral stroma in a radial distribution and then course anteriorly, running parallel to the ocular surface, forming the subbasal nerve plexus between the Bowman's layer and the basal epithelium.[1,8] Corneal innervation has important trophic functions and plays an important role in the regulation of epithelial integrity, proliferation, and wound healing.[1,3,6,8] Although herpetic keratitis is the entity most frequently associated with a decrease in the subbasal corneal nerve plexus and with neurotrophic keratopathy, other infectious diseases may be associated with corneal nerve loss as well.[4,9,11–14]

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Laser in vivo confocal microscopy (IVCM) is a noninvasive, high-resolution tool that allows imaging of the living cornea at the cellular level, providing images comparable with histochemical methods.[8,9,11] In recent years, the use of IVCM has revealed the importance of corneal nerves in both healthy eyes and ocular diseases, including IK, corneal transplantations, laser keratorefractive surgeries, neurotrophic keratopathy, and dry eye disease.[4,15,16] Using IVCM, we recently demonstrated a decrease in corneal nerves and an increase in immune dendritic cells in patients with microbial keratitis.[9,11] Although these findings may have significant clinical and surgical implications, no longitudinal studies have yet been performed in these patients to assess whether corneal nerve damage persists or resolves through reinnervation.

The aim of the present study was to investigate the longitudinal changes in subbasal corneal nerves in patients with *Acanthamoeba*, fungal, or bacterial keratitis beyond the treatment period. We hypothesized that corneal reinnervation takes place after the acute phase of IK. Herein, we describe the level of nerve degeneration in the acute phase of IK and regeneration after resolution of infection.

Methods

Patients

This was a prospective, longitudinal, single-center study conducted in a controlled, single-blinded fashion. Fifty-six eyes of 56 patients treated for acute IK at the Cornea Service of the Massachusetts Eye & Ear Infirmary, Boston, Massachusetts, between 2008 and 2014 were included in the study. Thirty eyes of 30 normal volunteers comprised the control group, of whom 18 were contact lens wearers. This study complied with the Health Insurance Portability and Accountability Act, adhered to the tenets of the Declaration of Helsinki, and was approved by the institutional review board/ethics committee of our institution. Written informed consent was obtained from all subjects after a detailed explanation of the nature of the study.

The diagnosis of acute IK was made by cornea specialists based on clinical history and ophthalmic examination. All patients and healthy controls underwent slit-lamp biomicroscopy. Only patients with positive corneal culture results or positive confocal findings for fungal or *Acanthamoeba* keratitis were included. The study excluded subjects with a history of any prior episode of IK, ocular inflammatory disease, ocular trauma, previous eye surgery, and diabetes.

In Vivo Confocal Microscopy

Laser scanning IVCM (Heidelberg Retina Tomograph 3 with the Rostock Cornea Module; Heidelberg Engineering GmbH, Heidelberg, Germany) of the central cornea was performed in all subjects. This microscope uses a 670-nm red wavelength diode laser source, and is equipped with a ×63 objective immersion lens with a numerical aperture of 0.9 (Olympus, Tokyo, Japan). The IVCM provides images that represent a coronal section of the cornea measuring 400×400 μm, which is 160 000 μm^2, at a selectable corneal depth and is separated from adjacent images by approximately 1 to 4 μm, with lateral resolution of 1 μm/pixel.

Digital images were stored on a computer workstation at 30 frames per second. A disposable sterile polymethyl methacrylate cap (Tomo-Cap; Heidelberg Engineering GmbH), filled with a layer of hydroxypropyl methylcellulose 2.5% (GenTeal gel; Novartis Ophthalmics, East Hanover, NJ) in the bottom, was mounted in front of the Rostock Cornea Module optics for each examination. One drop of topical anesthesia of 0.5% proparacaine hydrochloride (Alcaine; Alcon, Fort Worth, TX) was instilled in both eyes, followed by a drop of hydroxypropyl methylcellulose 2.5% (GenTeal gel) in both eyes. One drop of hydroxypropyl methylcellulose 2.5% also was placed on the outside tip of the Tomo-Cap to improve optical coupling and was advanced manually until the gel contacted the central surface of the cornea.

In vivo confocal microscopy was performed at 3 time points for each study eye: at the first visit to the cornea service (acute phase), at the day of cessation of antimicrobial treatment, and also at the last visit in the interval between 1 to 6 months after cessation of antimicrobial treatment (recovery phase). A total of 6 to 8 volume and sequence scans per time point were obtained from the center of each cornea, at least 3 of which were sequence scans with particular focus on the subepithelial area and the subbasal nerve plexus, typically at a depth of 50 to 80 μm. Sequence scans allow imaging the same area continuously, permitting the capture of large numbers of images of the subbasal nerve plexus per scan. Thus, the scans yielded 300 to 400 images of the subbasal layer alone. When a corneal ulcer was present with an epithelial defect, both the ulcer and the surrounding areas were scanned.

Corneal Sensation

Corneal sensation was measured in the central area of the cornea with the Cochet-Bonnet esthesiometer (Luneau Ophthalmologie, Chartres, France) in all subjects as previously described.[13] This test mechanically stimulates corneal nerves by touching the tip of a retractable 6.0-cm long monofilament nylon thread 0.12 mm in diameter against the corneal surface, decreasing in steps of 1.0 cm if a positive response was not obtained or advancing by 0.5 cm if a positive response was obtained. The longest filament length that resulted in a positive response was considered the corneal threshold.

Data Analysis

Three representative images were selected for analysis of each eye for each time point. The images were selected from the layer immediately at or posterior to the basal epithelial layer and anterior to the Bowman's layer by 1 experienced observer (R.T.M.). The criteria for selecting the images were the best focused and most complete images, with the entire image in the same layer, without motion, without folds, and with good contrast. Two observers (R.T.M. and F.A.), masked to the study groups and diagnoses, performed the IVCM image analyses. The nerve analysis was carried out using the semiautomated tracing program NeuronJ,[17–19] a plug-in for ImageJ (available at: www.imagescience.org/meijering/software). Nerve density was assessed by measuring the total length of the nerve fibers in micrometers. The main nerve trunk was defined as the total number of main nerve trunks in 1 image after analyzing the images anterior and posterior to the analyzed image to confirm that these did not branch from other nerves. Nerve branching was defined as the total number of nerve branches in 1 image. The number of total nerves measured was defined as the number of all nerves, including main nerve trunks and branches in 1 image (Fig 1).

Statistical Analysis

Statistical analysis was performed using Stata software version 13.0 (Stata Corp., College Station, TX). Continuous variables were expressed as mean ± standard error of the mean, whereas categorical variables were described by frequency and percentage, unless otherwise indicated. The distribution of data was