Architecture of Human Corneal Nerves

Linda J. Müller, Gijs F. J. M. Vrensen, Liesbeth Pels, Bob Nunes Cardozo, and Ben Willekens

Purpose. The corneal innervation, mainly analyzed in light microscopical studies, has been described as radially oriented stromal nerve bundles that ramify as leashes in the subbasal plexus. The current study aims to determine the orientation, the size, and the postmortem changes of the nerve fibers in the subbasal plexus of the human cornea.

Methods. Before processing for light and electron microscopy, the position of the corneas within the enucleated eyes of persons with melanoma and pairs of postmortem eyes was marked. The orientation and postmortem changes of the fibers were studied in serial "en face" semithin sections, and the size was determined in random, ultrathin cross-sections.

Results. Thirteen and a half hours after death, the majority of the nerve fibers were degenerated or gone. Nerve fiber bundles in the subbasal plexus run first in the 9-3 hours direction, then after bifurcation in the 12-3 hours direction and after a second bifurcation again in the 9-3 hours direction. From the main straight bundles, single-beaded fibers branch and run obliquely. Quantification of the nerve fibers shows an equally dense innervated central and central-peripheral cornea (mean fiber diameter, 0.4 μm) and a five to six times lower innervated peripheral cornea (mean fiber diameter, 0.67 μm).

Conclusions. The nerve bundles in the subbasal plexus of the human cornea form a regular dense meshwork with equal density over a large central and central-peripheral area. Because of their size, the majority of the fibers can be classified as C-fibers. Invest Ophthalmol Vis Sci. 1997;38:985-994.

The nerve fiber pattern of the densely innervated mammalian cornea has been described as radially oriented nerve bundles entering the corneal domain through the sclera. After passing Bowman’s layer, they ramify and end within the epithelium as free nerve endings. These nerve endings are derived from myelinated A-α and unmyelinated C-nerve fibers. In the skin, A-α and C-nerve fibers measure 1 to 5 μm and 0.2 to 2 μm, respectively. They are known to act as nociceptors, which monitor mechanical, thermal, and chemical signals in peripheral nerves including the cornea. In the rabbit, the density of corneal nerve endings is 20 to 40 times that of tooth pulp and 300 to 600 times that of skin. Although the human cornea is known to be extremely sensitive, the density of free nerve endings has not been estimated. This is mainly because corneal nerves degenerate rapidly after death. This is a disadvantage because discussions on corneal pain perception and spreading of neurotrophic viruses (Herpes) are more meaningful if the distribution of human corneal nerves would be known. In the past decades, numerous studies have been performed on transmitters occurring in corneal nerve bundles. They appeared to stain positive with antibodies against classical neurotransmitters, for instance acetylcholine and catecholamines, and antibodies, against peptidergic transmitters such as substance P and calcitonin gene-related peptide. The presence of classical and peptidergic transmitters may represent specific corneal nerve fiber subpopulations or may point at colocalization of different transmitters in the same fibers. Using different approaches, it became clear that in the central cornea, nerve fiber bundles located at the anterior border of Bowman’s layer run parallel to each other. Because the position of the corneas in the eye was not marked in these studies, it remained inconclusive whether nerve fiber bundles run temporally–nasally (9-3 hours), superiorly–inferiorly (12-6 hours), or obliquely. Recently, it has been shown elegantly by in vivo confocal microscopy that human cor-
FIGURE 1. (A) En face section of the central cornea (13.5 hours after death) at the level of the basal epithelial cells (*) and Bowman's layer (BL). Degenerating nerve fibers run parallel in the upper lower direction (arrow). (B) En face section of the fellow cornea (35.5 hours after death) at the same level as in (A). Notice the halos surrounding the epithelial cells (*). (C) Detail of (A) showing that the nerve fibers (arrow) have changed into strings of vacuoles. (D) Enlargement of B in which epithelial edema is visualized as halos surrounding the cells (*). The nerve fiber bundles (arrow) hardly can be distinguished among the epithelial cells. (E) Fixation within 1 hour after removal of the corneal button results in intact, straight running parallel organized nerve fiber bundles (arrow) in the central-peripheral cornea. (F) Enlargement of (E) shows connections (arrow) between the parallel running nerve fibers at the basis of the basal cells (*). BL = Bowman's layer; Ep = epithelium; N = nucleus. Bar = 0.2 mm (A,B), 0.1 mm (C,D), 0.2 mm (E), 0.05 mm (F).
neal nerve fiber bundles run temporally-nasally and beaded fibers obliquely. Furthermore, there is no information regarding their size and postmortem degeneration in the human cornea. The current study aims to determine the orientation, the size distribution, and the postmortem changes of the nerve fibers in the subbasal nerve plexus.

METHODS

Transmission Electron Microscopy

For measuring the size of the nerve fibers, seven corneas derived from eyes of people with melanoma (six approximately 1 hour after enucleation and one less than 15 minutes after enucleation) were used. The degree of anterior extension of the melanoma was rather small, indicating hardly any or no effect on the corneal nerves. Donor age varied between 49 and 87 years. Postmortem effects as well as orientation of the nerve fiber bundles were studied by comparison of two corneas derived from eyes of people with melanoma (one less than 1 hour after enucleation and one less than 15 minutes after enucleation) and two pairs of postmortem corneas, of which one was processed immediately. The fellow eye was left for 24 hours in a phosphate-buffered saline before fixation (first pair, 72 years old, fixed after 13.5 versus 37.5 hours after death and the second pair, 77 years old, fixed after 16.5 versus 40.5 hours after death) was used.
Because the cornea was 12.6 mm across, 4 mm of the central third was used as the central, the surrounding 2 mm as the central—peripheral, and the remainder as the peripheral part. Samples of approximately 4 mm² were dissected. Because of the curvature of the cornea, only 1 to 2 mm² of each sample could be used for analysis in "en face" sections. After fixation in 1.25% glutaraldehyde-1% paraformaldehyde in 0.08 M cacodylate buffer for several days, the tissue was postfixed in 1% OsO₄ supplemented with 1.5% ferrocyanide in 0.1 M sodium cacodylate buffer. The osmolality of the aldehyde fixatives was determined and kept within the range of 475 to 500 mOsmol. After being dehydrated in a graded series of ethanol, the pieces were flat embedded in epoxy resin. For en face sections, pieces of tissue were sawed off and mounted on epoxy resin blanks.

Postmortem changes and orientation of the nerve fiber bundles were analyzed in serial, Toluidine blue stained, 1 μm en face sections reaching from the anterior epithelium to the anterior stroma. At levels where structures of interest appeared in the light microscope, ultrathin sections (60 to 80 nm) were cut for transmission electron microscopy. To measure the size of the nerve fibers, photographs of randomly selected ultrathin cross-sections were used. Ultrathin sections were stained with uranyl acetate and lead citrate and inspected in Philips EM 201 and CM 12 electron microscopes (Philips Industries, Eindhoven, The Netherlands). Methods for securing human tissue were humane, and the tenets of the Declaration of Helsinki were followed.

Reconstruction

Nerve bundles in samples of the central, central—peripheral, and peripheral part of five corneas were reconstructed. At low magnification (×100), outlines of consecutive semithin sections were drawn on transparent paper using a drawing tube. At higher magnification (×260), nerve fibers were drawn and reduced to fit in the drawings obtained at low magnification. They were copied on transparent sheets and superimposed, thus reflecting the three-dimensional organization of the nerves. In addition, reconstructions of nerve fiber bundles at the ultrastructural level were made. The outlines of the profiles of the nerve fibers and the basal cells were drawn on paper, overlaying collages of photographs taken at a final magnification of ×7500.

Quantification

Nerve fiber profiles present in cross-sections of the different samples were photographed at a final magnification of ×31,000. Fifteen photographs of each specimen were made. With the Videoplan (Vidas Kontron, Munich, Germany), the minimal and maximal diameters of individual nerve fibers in the central (N = 714), central—peripheral (N = 640), and peripheral (N = 126) cornea were determined. In addition, the diameter of the nerve fiber bundles (N = 113) was measured. Nerve fibers, being considered as long tubes, will appear as circular profiles in cross-sections and as ellipsoid profiles in oblique sections. The small diameter of the ellipsoid profiles approximates the real diameter of the nerve fibers. Therefore, the minimal diameter was used as an estimate of the size of the nerve fibers.

Statistical Analysis

A one-way analysis of variance test was used to determine differences in frequency distributions of the nerve fiber diameter. Differences were considered significant when P < 0.05.
to each other in the zone between Bowman's layer and the basis of the basal cell layer. It became clear that nerve fiber bundles, sometimes forming loops, are interconnected (Figs. 1E, 1F). However, in light microscopy, it is impossible to establish the exact posi-

FIGURE 4. (A) Low power electron micrograph of a nerve fiber bundle in the subbasal plexus containing a bead at the periphery (arrow). (B) Enlargement of the bead in (A) illustrating that the bead is located within the bundle of straight nerve fibers. It contains an accumulation of mitochondria (M) and glycogen particles (arrow). All the nerve fibers contain vesicles (arrowheads). Ep = epithelium. Bar = 2.5 μm (A), 1 μm (B).

RESULTS

Light Microscopy

Analysis of central, central-peripheral, and peripheral en face sections showed that subbasal nerve bundles, closely aligned to Bowman’s layer, show signs of degeneration within 13.5 hours after death, as shown in Figure 1A. At higher magnifications, these nerve bundles have changed into strings of vacuoles (Fig. 1C). In the fellow cornea (37.5 hours after death), degeneration was even more pronounced; at low magnifications, it was difficult to distinguish the nerve fiber bundles among the swollen epithelial cells, which are surrounded by lucent halos (Fig. 1B). However, at higher magnification, the vacuolized nerve bundles were identifiable (Fig. 1D). Postmortem alterations in the epithelium were more pronounced in the second pair of eyes (16.5 versus 40.5 hours after death, not illustrated).

In corneas fixed within less than 1 hour after enucleation, nerve fiber bundles proved to run parallel

FIGURE 5. (A) A cross-sectioned nerve bundle containing seven profiles with circular profiles. Notice the mitochondria (⋆) and glycogen particles (arrow). (B) A single oval-shaped nerve fiber profile in the subbasal plexus filled with mitochondria. (C) This cross-sectioned nerve bundle shows profiles with varying diameters and shapes. Lines on the profiles in (A,B) and (C) represent the minimal diameter. BL = Bowman's layer; Ep = epithelium. Bar: 1 μm (A,B,C).
tion of the nerve plexus within the cornea. Reconstructions of electron micrographs (Fig. 2A) showed that the outlines of the basal cells are irregular in form and that the nerve fiber bundles, consisting of four to six nerve fibers, run between the basal cell layer and Bowman’s layer. A drawing of the reconstruction in Figure 2A illustrates the highly undulated membranes of the basal cells (Fig. 2B).

Reconstructions of nerve fiber bundles in postmortem and fresh tissue at the light microscopic level showed that the main bundles run in parallel. Despite the presence of many degenerated fibers in postmortem corneas, this pattern was not abolished. Part of one of the reconstructions derived from a cornea of which the position was marked in the eye is shown in Figure 3. This figure illustrates that the main large bundles run in the 9-3 hours direction (Fig. 3A). From these nerve bundles, smaller intermediate bundles bifurcate, which run at almost right angles in the 12-6 hours direction. From the intermediate bundles, smaller bundles bifurcate and run again in the same direction as do the large main bundles (9-3 hours) (Fig. 3B). Nerve fiber bundles consist of straight fibers and beaded fibers (Fig. 4A). The beads are located at the periphery of the bundle and are characterized by an accumulation of mitochondria and glycogen particles (Fig. 4B).

Quantification
Cross-sections through the central, central-peripheral, and peripheral cornea show nerve fiber bundles (Fig. 5A) and large single nerve fibers (Fig. 5B) at the basal aspect of the basal cells. When a nerve bundle is cut at almost right angles, it contains more or less circular profiles as illustrated in Figure 5A. However, when the bundle is hit obliquely, the nerve fiber profiles appear as oval shapes. In general, microtubules dominate in the small fibers and neurofilaments in the large fibers (Fig. 5C). Numerous mitochondria and clusters of glycogen particles are present in large fibers (Figs. 5A and 5B).

The distributions of nerve fiber diameters are shown in Figure 6 for the central (Fig. 6A), the central-peripheral (Fig. 6B), and the peripheral part (Figs. 6C, 6D) of the cornea. The distributions are skewed at the right side. In the periphery, the tail appeared to be rather long, and therefore it is presented in a separate graph (Fig. 6D). Nerve fibers vary in size between 0.03 and 2.5 μm, and the majority of the fibers is between 0.1 and 0.5 μm. The average size in the center is 0.36 μm ± 0.18 (mean ± standard deviation), in the center-periphery 0.34 μm ± 0.2, and in the periphery 0.67 μm ± 0.64. Equal numbers of photographs showed that the number of nerve fiber profiles encountered in the center (N = 714) and in the center-periphery (N = 640) is approximately five to six times higher than the number encountered in the periphery (N = 126). No significant differences were observed between the distributions in the center and in the center-periphery (P < 0.01), whereas both distributions differed significantly from the distribution in the periphery (P < 0.01).
because approximately one fourth (32 fibers) of the peripheral fibers are larger than 1.3 \( \mu m \) (Fig. 6D). The distributions of the nerve bundle diameters also were skewed and had an average size of 1.0 ± 0.3 \( \mu m \) (data not shown).

**DISCUSSION**

**Architecture of Human Corneal Nerves**

The current study shows that 13.5 hours after death, a great part of the human corneal nerves has degenerated or gone. As analysis of the nerve fiber distribution in the subbasal plexus was carried out with corneas obtained from persons with melanoma, the question arises whether this distribution reflects the real architecture of human corneas. It has been stated that corneal nerves become more prominent in corneas with an intrinsic corneal disease. Examples are Fuch’s dystrophy, bullous keratopathy, keratoconus, posterior polymorphous dystrophy, and herpes zoster–herpes simplex keratitis. For corneas obtained from patients with melanoma, such a prominent increase has not been reported. Analysis of these corneas showed that the parallel organization of the main nerve bundles was similar to that observed in in vivo confocal microscopy. Therefore, the conclusion seems justified that the pattern of the nerve tree in corneas of patients with melanoma reflects the real architecture of human corneas.

Light and electron microscopic observations gave a precise description of the penetration of nerve fiber bundles into the corneal domain from the limbal area. Because of quantitative measurements, it became clear that the branching pattern in rabbits, as described by Rózsa and Beuerman, is different from that in human. In rabbit, the number of intersections decreases gradually from the center toward the limbus, whereas the current study does not provide evidence for such a gradual decrease in the number of nerve fibers. This number, given as an arbitrary measure of profiles per 15 photographs, is five to six times less in the periphery than in the center—periphery. Comparison of our data with those of the rabbit suggests that the apex of the rabbit cornea is innervated heavily, whereas the central two thirds of the human cornea equally is dense innervated. These data have led to the following architecture of the corneal subbasal plexus in humans. Radially oriented thick stromal nerve bundles, running parallel to the corneal surface, enter the corneal domain from the limbus. Before penetration of Bowman’s layer, stromal nerve bundles bend 90°. Subsequently, the nerve fiber bundles bend again 90° and run between the basal cell layer and Bowman’s layer in a 9-3 hours direction. From these bundles, a rather regular meshwork of finer branches is formed, as illustrated in Figure 7. As described in a previous article, in cross-sections, alternating nerve fiber bundles and single fibers are located at more or less equal distances. The current study, based on en face sections, supports the previous observations and shows connections of main nerve bundles with obliquely running single-beaded fibers. A three-dimensional artist’s impression of the distribution of the nerve fibers is given in Figure 8. The large nerve fiber bundles entering the subbasal plexus consist of straight fibers and beaded fibers. The straight nerve fiber bundles splice and also bifurcate several times to become gradually smaller nerve bundles. From the main large bundles, single-beaded fibers bifurcate and run obliquely. At certain locations of the meshwork, these beaded fibers turn 90° in an upward direction (Fig. 8). This conclusion is based on observations that large profiles (with many mitochondria), approximately two to four times larger than those of straight nerve fibers, were present above the basal cell layer in en face sections. The reason for such a specific organization is not known, but one can imagine that in this way, a homogeneous distribution of nerve endings over the central and central–peripheral cornea is guaranteed.

To provide us with the resolution of this meshwork, it is important to know the distances between the intermediate and the smaller nerve fiber bundles.
FIGURE 8. Three-dimensional drawing of the penetration and distribution of stromal bundles into the subbasal plexus. The unmyelinated nerve fiber bundles (blue) are sometimes splitting, have bifurcations at almost right angles, and consist of several straight (red) and single-beaded (green) fibers. Single-beaded fibers bifurcate obliquely and turn upward between the basal cells to reach the wing cells.

However, measuring distances in a significant sample requires many more fresh human corneas and is a tedious, almost impossible, task. In vivo confocal microscopy could give a rapid impression, but the resolution of images in whole-mount tissue in comparison to those in thin sections still is poor, so that intermediate and small nerve fiber bundles will not be observed easily. What can the meshwork tell us about the local sensitivity of pain perception? The density in the cornea is known to be rather high in comparison to tooth pulp (20 to 40 times) and extremely high in comparison to the skin (300 to 600 times). The distance between the first-order branches is in the order of magnitude of 20 to 50 μm, and between the second and third branches, it certainly will be smaller, indicating that injuries to individual epithelial cells may be sufficient to give a pain perception.

The parallel organization of the nerve fiber bundles described here seems to be specific for the human cornea because it also was observed in formaldehyde solution-fixed tissue stained with hematoxylin, in gold chloride-impregnated tissue, and in in vivo confocal microscopy. In gold chloride-impregnated epithelial tissue, Schimmelpfennig has described fusion of nerve fibers. A fusion in Schimmelpfennig's study is described as a branching point in in vivo confocal microscopy. These fusions or branching points also were observed by Martinez in formaldehyde-fixed tissue. It is tempting to speculate on fusion of nerve fiber bundles, because single fibers fuse with small bundles to become gradually thicker bundles. Because several figures in the aforementioned studies and in the current study show that thick bundles divide into two smaller ones of the same size and then fuse again to become a bundle of the original thickness (Fig. 1F), it seems rather unlikely to describe the branching points as fusions. The bundles most likely splice to increase the surface area of the sensory receptors. In addition, in this respect, most light microscopic descriptions of nerve fibers are nerve fiber bundles when studied with the electron microscope. Only at the location of the beads are single fibers clearly visible in light microscopy.

A quantitative analysis as performed in the current study has not been carried out previously. In mice, an attempt has been made to measure the size of the individual fibers, but because of variations in size, it was difficult to draw conclusions. Despite the great variation and poor quality of the tissue, it seems that in mice, most of the nerve fibers measured are 0.4 μm.
Architecture of Human Corneal Nerves

or less. This size is in the range of the physiologically classified C-fibers (0.2 to 2 μm) and not in the range of the Aδ nerve fibers (1 to 5 μm) in the skin. The current study suggests that the majority of the nerve fibers in the human cornea are also C-fibers. In the periphery, the size of the nerve fibers does not allow a discrimination between C and Aδ fibers because many are equal to or larger than 1 μm across, which means that they can belong to both classes. In addition, single-beaded fibers can not be classified simply because they have the same size as that of large straight fibers at the location of the beads. Although no quantitative data on the size distribution of the stromal nerve fibers are available, both small (0.4 μm) and large fibers (1 μm), as observed in the subbasal plexus, also are present in the corneal stroma. The size of the nerve fiber bundles is taken into account, the number of fibers larger than 1 μm (32 + 113 μm) is four times higher. This figure may be in favor of the Aδ fibers, but still this number is not enough to explain the distribution of type I (thermal and chemical) and type II (mechanoreceptive) endings as described by Maclver and Tanelian. The skewed frequency distributions of the minimal diameters with a tail at the side of the larger diameters indicate two populations of fibers. However, the tail is too small to distinguish two overlapping distributions. The small tail might be related to the relatively low number of beads hit in the sections. Therefore, it is concluded that the skewed distribution in the center and center-periphery probably is caused most by beads and in the periphery by large fibers.

Density of Nerve Fibers

The density of nerve fibers in human corneas after peptidergic and classical neurotransmitter stainings as found in light microscopic studies is difficult to compare with the density of nerve fibers in the current study and that of Schimmelpfennig. Analysis of rapidly fixed fresh corneal tissue and of gold chloride-treated corneal tissue shows larger numbers of corneal nerves. Differences in fiber density in human corneal tissue only can be ascribed partly to the specificity of the antibodies or to penetration of the antibodies used in immunocytochemical stainings. An additional factor influencing the density of immunostained nerve fibers might be the quality of the tissue. It is known that postmortem eyes lose their epithelium easily after death (Cornea Bank Amsterdam, personal communication, 1996). In addition, this criterion does not explain the differences in density because Usitalo et al28 have observed beads after enucleated eyes and Ueda et al29 have used postmortem corneas. Usitalo et al28 have observed these beads in the center and center-peripheral area. Because of their size, the majority of the fibers can be classified as C-fibers.

Key Words

epithelium, human corneal nerve fibers, quantification, subbasal plexus, ultrastructure

Acknowledgments

The authors thank Drs. Jan Klooster and Jan Ruijter for discussions; Niko Bakker, Ton Put, and Marina Danzman for the drawings and photographs; Drs. M. Kliffen and C. M. Mooy at the Department of Ophthalmology, Erasmus University, Rotterdam; and L. Koornneef at the Academical Medical Center, Amsterdam, for their efforts in obtaining fresh corneal tissue derived from eyes of persons with melanoma.

References


