A Cryodamage Model for Studying Corneal Nerve Regeneration

Kwan Y. Chan, Markeita Järveläinen, Jean H. Chang, and Michael J. Edenfield

This study used a transcorneal freezing technique to produce a 2-mm circular, central wound in the rabbit cornea for investigating corneal nerve regeneration. All the corneal cells, nerves, and associated Schwann cells were dead inside the wound, but the extracellular matrix components remained intact. The destroyed epithelium and endothelium were replaced in 1 and 5–7 days, respectively. The necrotic keratocytes and stromal and subepithelial nerves were removed completely in 1–3 days by invading macrophage-like cells. The wounded stroma was repopulated centripetally by migrating keratocytes between days 1–5. Two types of nerve growth were identified in the stroma. The first type was novel sprouting of straight, long neurites between days 2–21, initially from the undamaged, periwound nerves and later from regenerated stromal nerves inside the wound. These small-caliber neurites proliferated in a random and disorderly pattern both inside and outside the wound and sometimes terminated on stationary, stellate keratocytes. The second type was genuine regrowth of stromal and subepithelial nerves in a centripetal direction between days 3–7. Schwann cells appeared on the newly formed nerves starting on day 4 or 5. A near-normal pattern and size of the nerves were established in the wound as early as day 10. In the epithelium, transient, wound-oriented neurites (days 1–3), single nerves, and semileashes (days 4–10) appeared. A near-normal leash pattern was restored between days 10–21 only at the wound periphery. Thus, in this model, the major groundwork of nerve regeneration occurred between days 3–10, simultaneously, at all three levels of nerve organization. These data suggest that nerve–Schwann cell interaction contributes to the restoration of stromal and subepithelial nerves, whereas a reparative epithelium deficient in trophic activity may account for the incomplete regrowth of epithelial nerves. The cryodamage model offers an efficient and multifaceted system for the experimental study of corneal nerve regeneration.


The innervation of the mammalian cornea consists of three levels of organization: stromal, subepithelial, and epithelial nerves. Damage to corneal nerves may result from various disease processes (e.g., herpetic keratitis), chemical or mechanical injuries, and ocular surgery (e.g., penetrating keratoplasty). Numerous clinical studies based on corneal sensitivity measurement or postmortem examination of grafted corneas have shown that nerve regeneration in the human cornea is a slow process which can take months or years. The rate of recovery of tactile sensitivity in various diseased corneas was measured to average 0.029 mm/month. The pattern and density of the regenerated nerves are seldom restored completely.

In the past, animal studies of corneal nerve regeneration used mechanical or surgical wounding techniques to damage the corneal nerves, such as keratectomy and perilimbal incisions. In these studies, various degrees of nerve regeneration were observed in 30 days (keratectomy) or 30 months (perilimbal incision). Recently, we used the heptanol technique to produce a well-defined corneal wound deprived of epithelium and intraepithelial nerves. In this model, partial regeneration of the epithelial nerves is completed after 3–4 weeks with no further improvement up to 10 weeks after wounding. There is a need to develop an experimental model for studying corneal nerve regeneration at all three levels of nerve organization more efficiently. We report a new model based on a transcorneal freezing technique in which the regrowth of stromal, subepithelial, and epithelial nerves occurs 3–10 days after wounding.

Materials and Methods

Cryodamage Model

Male New Zealand albino rabbits (2.0–2.5 kg) were anesthetized by subcutaneous injection of a mixture of ketamine (60.0 mg/kg) and xylazine (2.4 mg/kg) solutions. All experiments with these animals were
done in accordance with the ARVO Resolution on the Use of Animals in Research. The rabbit was laid on its side, with one eye proptosed and rinsed with sterile saline solution. A steel cryoprobe unit (Kryospray II; Brymill, Vernon, CT) with a 1-mm diameter tip was purged internally with liquid nitrogen to \(-60^\circ\text{C}\) (precalibrated) and brought into direct contact with the center of the corneal surface for 15 sec. The frosted area was immediately irrigated with sterile saline (ambient temperature) until clarified. The same procedure was repeated on the opposite eye. After wounding, the eyelids were kept closed by applying surgical tape until the rabbit regained consciousness. The eyes were examined daily for 1 week for signs of inflammation and discomfort. Two or three rabbits were killed on days 0, 1, 2, 3, 4, 5, 6, 7, 10, 14, and 21 after wounding. We used 27 rabbits in this study.

Tissue samples of unwounded corneas from previous studies\(^7\)\(^8\) were used as the control.

**Histologic Techniques**

The eyes were enucleated after the rabbits were killed, and the superior limbus was marked with a small needle. The corneas were excised, and their orientation was tracked in all subsequent steps by making appropriate small cut marks. In some cases, the cornea was placed in phosphate-buffered saline and cut into halves along the midline of the wound (which showed as a hazy or translucent spot up to 6 days after wounding). One half of these corneas was stained with gold chloride, and the other half was embedded in methacrylate. In other cases, the whole cornea was stained with gold chloride.

Gold-chloride staining of the corneal nerves (including fixation techniques) was done according to Rozsa and Beuerman,\(^1\) as described previously.\(^7\) After staining, the corneas were trimmed to 5-mm square pieces containing the wounded area. The tissues were placed in 30% sucrose solution overnight (until they sank). Sections were cut parallel to the corneal surface using a freezing microtome (80-\(\mu\)m). Sections were cut parallel to the corneal surface using a freezing microtome (80-\(\mu\)m) and were a monolayer on day 1. However, by day 2, the orientation of the cornea showed a cell-free, circular area in the stroma measuring 1.8-2.0 mm in diameter 1-2 days after wounding. Transverse sections of the wounded area showed disappearance of keratocytes in the anterior half of the stroma on day 1 (Fig. 1A), which extended to the posterior stroma by day 2 (Fig. 1B). By day 7, the stroma and the keratocyte population appeared normal (Fig. 1C).

One day after cryodamage, the destroyed epithelium in the wounded area was replaced, possibly by migrating cells from the wound margin. Some polymorphonuclear leukocyte-like cells (3-12 \(\mu\)m) were present in the basal region of epithelium (Fig. 1D). The damaged endothelial cells in the wounded area were a monolayer on day 1. However, by day 2, the endothelium was multilayered in the center of the wound (transverse section), suggesting hyperplasia (Fig. 1B). Enface sections (gold-chloride staining) showed cell aggregation in the wounded endothelium, similar in appearance to that observed by scanning electron microscopy.\(^9\) The multilayer appearance persisted even at day 7, when the rest of the wounded area resumed normal morphology (Fig. 1C). The cells in the multilayers appeared epithelioid and similar in size to normal endothelial cells (Fig. 1E). In some wounded corneas, normal endothelial morphology (a mosaic monolayer) was restored as early as day 5.
Morphologically, the stroma underwent the most changes after wounding. Necrosis and complete removal of the cryodamaged keratocytes in the wounded area took place during the first 2 days. Macrophage-like cells, that could be responsible for the removal of dead cells and damaged nerves, were present in the wounded stroma between days 1–7 (peak, days 3–4; Table 1). The gold-chloride technique not only stained the nerves as dark structures but also stained different cell types in various degrees of orange, red, and dark colors (no nuclear or membrane staining). The macrophage-like cells were stained dark by gold chloride, with various dark granules in the cytoplasm (Fig. 2A). Some of these cells showed dark, narrow stripes in the midregion of the cell (Fig. 2B). The shape of the macrophage-like cells varied from spheric (20–26 μm in diameter) to epithelioid (9–10 μm by 36–54 μm) to tadpole-shaped (13–15 μm wide at one end and tapered to a length of 50–60 μm) (Fig. 2A). There was a mass migration of surviving keratocytes from the wound margin towards the center of the wound, starting on day 1 and reaching the center on days 4–5 (Figs. 2C–D). The morphology of these migrating keratocytes was distinctly different from normal stationary cells. The former cells (stained reddish-orange) had one to three parallel lobes (2–5 μm wide, 100–200 μm long), connected with the soma (Fig. 2E). The long axis of the cells was oriented at different angles to the center of the wound. The overall appearance of the migratory zone of keratocytes in en face section was splinterly (Fig. 2F). In contrast, the normal stationary keratocytes appeared stellate (20–30 μm in diameter) in en face section, with numerous short processes radiating outwards (Fig. 2G). After day 5, keratocytes in the repopulated wound region resumed the normal stellate morphology. At about this time, a ring of darkly stained, slender, spindle-shaped keratocytes (2–3 μm greatest width, 60–80 μm long) was observed at the wound periphery (Fig. 2H). This peripheral ring of cells did not change its localization even after 21

Fig. 1. Transverse sections of cryodamage wound in rabbit cornea (hematoxylin and eosin staining). (A) Day 1: The anterior stroma (above the chain of stars) is devoid of keratocytes starting from the wound margin (two-way arrow; the wound is to the right). The epithelium (ep) is already regenerated, and the damaged endothelium (arrow) remains a monolayer. (B) Day 2: most of the stroma (st) in the wound is devoid of keratocytes (above the chain of stars). The endothelium is multilayered in the center of the wound (between the arrows). (C) Day 7: the stroma (st) has restored its normal keratocyte population, but the endothelium remains multilayered in the center (between the arrows). (D) Polymorphonuclear leukocyte-like cells (arrows) are present in the basal layer of epithelium (ep) (day 1). (E) Detailed view of the endothelium at the transition between monolayer (small arrow) and multilayer (large arrow). Note the intact Descemet's membrane (arrowhead) (day 2). Calibration bar is 50 μm (A, also applies to B, C) or 20 μm (D, also applies to E).
Table 1. Chronology of morphologic changes in the cryodamage model

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Data base was four to six corneas.
+ = present or occurring; (+) = observed in one cornea only; wp = wound periphery; wc = wound center; ← = centripetal direction; * = more in number.

days. During week 1, the cell density in the wounded stroma gradually increased (approximately one-half normal on day 4 and two-thirds normal on day 6), and it was close to normal after 21 days. The visual difference in the cell density of the wounded stroma compared with the periwound stroma in enface sections (Fig. 2D) provided an unequivocal localization of the wound region during the entire experimental period.

The following sections describe in detail the morphologic changes in the corneal nerves in the wounded corneas. Table 1 summarizes all the major findings chronologically.

**Stromal Nerves**

Stromal nerves are normally found in the anterior half of the stroma. In the wounded area, stromal nerves were absent during the first 2 days after wounding (Fig. 2C). Some necrotic segments of the nerves and necrotic Schwann cells were weakly stained by the gold-chloride technique at that time, (similar to Fig. 6A). Two types of nerve growth were identified in the wounded stroma (Table 1). The first type was random sprouting of new neurites previously not present in normal cornea. These neurites were generally straight, long, and very small in diameter (0.5 μm). They branched off from the undamaged nerve trunks close to the wound margin, emerging from either the distal stump or anywhere along the shaft of the trunk (Figs. 3A, 5C). The neurites grew into the wound area, lacking any specific direction beginning on day 2, peaked between days 6–7, and were still present up to day 21. The neurites were unique in that they ran long distances in a straight course and would abruptly turn and then continue in a straight course in the same focal plane (Fig. 3B). They could be traced for long distances (0.5–3 mm) across the wound in the same enface section (Fig. 3C). Beginning on day 6, some of the neurites also grew outside the wound (without specific direction). Occasionally, this happened as early as day 2 or 3. Some of the neurites (whether inside or outside the wound) appeared to terminate on or very close to the
Fig. 2. Enface sections of cryodamage wound showing changes in the stroma (gold chloride staining). (A) Macrophage-like cells are spherical (arrowheads), epithelioid (large arrows), or tadpole-shaped (small arrow) (day 2). (B) Detailed view of spherical (arrowhead) and epithelioid (arrow) macrophage-like cells showing the internal stripes (day 3). (C) A zone of migratory keratocytes is present between the wound margin (circle of white dashes) and the keratocyte-free stroma (circle of black dashes) in the wound (* indicates the center). The migratory zone is less darkly stained than the periwound stroma due to a lower cell density. Note the abrupt termination of undamaged stromal nerves (arrowhead) at the wound margin (day 2). (D) Most of the wounded stroma (encircled by arrowheads) is repopulated with migratory keratocytes except in
surface of stellate keratocytes (20–30 μm in diameter). Careful depth focusing of the surrounding structures immediately above or below the neurite termination point showed that the neurite was not broken at the sectioning plane but, rather, that it ended naturally in a beaded form (1–2 μm) (Fig. 4). The association of straight neurites with the keratocytes was first observed on day 3 (occasionally as early as day 2) and continued up to day 21, with the most observed between days 14–21. Between days 5–10, when stromal nerves were regenerated in the wounded area, the straight and long neurites also branched off from the regenerated nerves. Schwann cells were never associated with the straight neurites.

The second type of nerve growth was genuine regeneration (Table 1). Small- (0.5–1 μm) and medium-caliber (4–10 μm) nerve fibers regrew sequentially from the large-caliber (15–30 μm) nerve trunks at the periphery of the wound by day 3 (Fig. 5A) and reached the center of the wound by day 7 (Fig. 5B). The regenerated nerve trunks restored their normal size starting at the wound periphery on day 6 and finished at the center of the wound by day 10 (occasionally by day 14 or 21). During the initial few days of stromal nerve regrowth, the individual nerve fibers were bare and separated in the nerve bundle, which appeared shredded (Figs. 3A, 5D). From day 5 onwards, Schwann cells began to appear and align themselves linearly (4–8 μm wide, 50–70 μm long) on the nerve fibers or attach to the nerve branch junctions (14–18 μm wide) (Fig. 5C). The cells were long and slender (linear location) or dendritic (branch location) in shape, and stained light to medium-dark with gold-chloride impregnation (similar to Fig. 7B, C). Their increased presence and possible ensheathment of axons restored the normal compact, dark appearance and large size (18–30 μm) of the stromal nerve bundles and branch junctions (triangular shape) (Fig. 5E).

Subepithelial Nerves

The subepithelial nerves normally form a loop-like plexus in the most anterior stroma, 20–30 μm beneath the epithelium. In the cryodamaged area, the subepithelial plexus was completely absent during the first 3 days after wounding (similar to Fig. 2C), except for necrotic fragments stained lightly by gold chloride (Fig. 6A). The novel sprouting of straight and long neurites also occurred in this location, similar to those of the stromal nerves (Table 1). The neurites branched off from the undamaged nerve plexus next to the wound margin on day 2, initially grew into the wound area (Fig. 6B), and later also grew directly into the unwounded area by day 7 (occasionally as early as day 2). Later as the subepithelial plexus was regenerating inside the wound, similar neurites also sprouted from the regenerated nerves (Fig. 6C). The termination of some straight neurites on keratocytes located in the same subepithelial level of the stroma was also observed, beginning on day 4. All the neurites persisted up to day 21.

Regrowth of the subepithelial plexus began on day 3 from the wound periphery (Fig. 6D) and reached the center by day 7 (Table 1). Initially the nerve fibers were small in caliber (0.5–1 μm) and formed partial loops only. Later, beginning on day 7, the nerves appeared larger (2–4 μm caliber) and formed more loops (Fig. 6E). As early as day 10, a complete nerve plexus network (4–8 μm caliber) was regenerated throughout the wounded area (Fig. 6F). Occasionally, the completion took 14 or 21 days. Schwann cells were first observed on day 4 to align linearly (7–12 μm wide, 70–100 μm long) on the growing nerve fibers, spreading from the proximal to the distal part with time (Figs. 7A–B). Additionally in some locations on days 5 and 6, a group of three or more Schwann cells appeared to form a plexus-like track preceding the advancement of the growing nerve tip (Fig. 7C). In a fully regenerated subepithelial nerve, the trunk was stained dark and the branch junction covered with Schwann cells (Fig. 7D).

Epithelial Nerves

The epithelial nerves normally emerged from the subepithelial plexus as leash-like branches diverging from a common initiation point, running parallel to the basal cell layer (2–4 μm spacing between branches, occupying an area 20–25 μm wide and 80–120 μm long), and continuing into the anterior epithelial layers as wavy, free nerve endings. In the wounded area, epithelial nerves were absent during the center (encircled by black dashes) (day 3). (E) In the migratory zone, some keratocytes are stellate in shape (arrowheads), and others are transformed into migratory cells containing elongated processes (1–4; large arrow points to cell body; small arrow points to processes). Cell 2 has three parallel-oriented lobes (small arrows). Note the different orientation of cells 2, 3, and 4. The wound center is toward the lower left corner (day 2). (F) The splinterly appearance of the migratory zone (outside the semicircle of stars) is caused by the different orientation of the migratory keratocytes. Macrophage-like cells (arrows) are easily detected in the keratocyte-free region of the wound (the center is indicated by *) (day 2). (G) The normal stellate appearance of stationary keratocytes (arrowheads) outside the wound (day 2). (H) In the wound periphery, some keratocytes are spindle-shaped (arrows) instead of stellate-shaped (arrowheads), suggesting an edge-on orientation of stellate cells (day 10). Calibration bar is 20 μm (A, also applies to B, E, G, H), 0.5 mm (C, D), or 50 μm (F).
Fig. 3. Enface sections of cryodamage wound showing novel sprouting of straight neurites in the stroma (gold chloride staining). (A) In the wound periphery, a stromal nerve (arrowhead) appears shredded in the distal stump (large arrows), from which straight, long neurites (small arrows) branch out. Note the presence of macrophage-like cells (*). The wound center is toward the right (day 4). (B) Several straight, long neurites (arrows) are shown in this field, one with a sharp turn in direction (arrowhead) and an abrupt angle of branching (*) (day 4). (C) The crisscross pattern of straight neurites (small arrows) contrasts with a regenerating stromal nerve (arrowheads). Note the smaller diameter at the distal part of the nerve (large arrow) (day 5). Calibration bar is 50 μm.

The first 3 days (Table 1). An exception was that in some wounded corneas, parallel, radially running neurites sprouted into the regenerated epithelium from the undamaged subepithelial plexus surrounding the wound. These wound-oriented neurites (10–20 μm spacing between branches, in an area 60–100 μm wide by 120–180 μm long) were present only in the wound periphery and disappeared after 3 days (Fig. 8A). Occasionally, some parallel neurites were still present at day 6 or 14. The straight, long neurites were not found in the epithelium at all. By day 4, two types of nerve regrowth began. The first type was single nerves, originating directly from the subepithelial nerves at the wound periphery and later from the regenerated subepithelial plexus (Fig. 8B). These nerves took a wavy course into the upper epithelial layers. The second type was incomplete, semileashes (with two to three instead of four to six branches) which appeared in the wound periphery on day 4 and in the center of the wound on day 10 (Figs. 8C–D). Both types of nerves persisted up to day 21. A near-normal leash morphology was restored only in the wound periphery on day 10 (Figs. 8E–F). Up to day 21, the center of the wound was still lacking in normal leashes.

The normal leash pattern of the epithelial nerves is oriented towards the nasalmost limbus, with the branches pointing straight toward that direction. In the wounded epithelium, there was a tendency for the distal end of the semileashes and leashes to curve towards the center of the wound instead, especially at the nasal aspect of the wound (Figs. 8D–E). This unique feature was also observed and discussed in a previous study.8

Discussion

In the past, the technique of cell cryodamage was used to produce corneal wounds for the experimental study of various corneal cell functions9–11 or to perform cryotherapy for conjunctival melanoma.12 In this study, we extended the application of the cryodamage wound in the cornea to the experimental study of nerve regeneration. Transcorneal freezing killed all the corneal cells, nerves, and associated Schwann cells inside the frosted zone.13 The destroyed epithelium and endothelium were replaced in 1 or 5–7 days, respectively. The necrotic keratocytes and stromal and subepithelial nerves were removed completely within 2–3 days, possibly being phagocytosed by invading macrophage-like cells and other inflammatory cells. The extracellular matrix components in the wound were retained, including the stromal matrix, epithelial basal lamina, and Descemet’s membrane. The wounded stroma was repopulated centripetally by migrating keratocytes between days 1–5. But the normal cell density was not fully restored by day 21. Two types of nerve growth were identified in this model. From days 2–21, novel
spouting of straight, long neurites (first type) occurred in both stromal and subepithelial nerves, initially from the undamaged nerve fibers surrounding the wound, and later from the regenerated nerves inside the wound. These neurites proliferated inside and outside the wound without any specific direction. Some of the neurites terminated on stationary, stellate keratocytes. Genuine regrowth of stromal and subepithelial nerves (second type) occurred centripetally between days 3–7. Schwann cells appeared on the newly formed nerves starting on day 4 or 5. A near-normal pattern and size of the stromal and subepithelial nerves were established in the wound as early as day 10. Regeneration of the epithelial nerves was slower. Transient sprouting of parallel, wound-oriented neurites occurred sporadically in the epithelium during the first 2 weeks. Regrowth of the basoepithelial leash nerves took place centripetally between days 4–10 in the form of single nerves and semileashes. A normal leash pattern was restored in the epithelium between days 10–21 only at the wound periphery. In summary, the major groundwork of nerve regeneration in the cryodamage model occurred between days 3–10, simultaneously at all three levels of nerve organization. At day 21, the cellular and nerve organization of the wounded cornea was nearly normal, except for the lack of basoepithelial leash nerves at the center and the persistence of straight, long neurites in the stroma.

The cryodamage model offers several advantages that are lacking in all previous models. First, the time course of panocular nerve regeneration is shortened to 10 days instead of 4 weeks (epithelial nerves; keratectomy or heptanol wound) or 3–30 months (pancorneal nerves; perilimbal incision). The onset of regrowth starts on day 3 for both stromal and subepithelial nerves (instead of day 60 and 30, respectively, in perilimbal incision) and on day 4 for epithelial nerves.
Fig. 5. Enface sections of cryodamage wound showing genuine regeneration of stromal nerves (gold chloride staining). (A) A stromal nerve (large arrows) has regenerated partially into the wounded stroma (encircled by arrowheads). Note the smaller diameter at the distal ends (small arrows). Part of a regenerating subepithelial plexus is also shown (*) (day 6). (B) Large-caliber stromal nerves (arrows) are regenerated in the central region of the wound (circle of arrowheads indicates the wound margin) (day 7). (C) Medium-stained Schwann cells (large arrows) are present on the proximal part (large arrowheads) of a regenerating stromal nerve and absent on the distal new branches (small arrowheads). Note the numerous straight neurites (small arrows) derived from this stromal nerve (day 6). (D) The formation of branch junction (large arrows) from a regenerating stromal nerve (arrowhead). Schwann cells are absent in the junction at this time. The distal end of the nerve is indicated by small arrow (day 5). (E) Restored morphologic features of a regenerated stromal nerve. The branch junction and the main trunk present a smooth and compact appearance due to a covering of darkly stained Schwann cells (eg, next to *). The proximal (arrowhead) and distal (arrows) parts are distinguishable (day 10). Calibration bar is 0.5 mm (A, B) or 50 μm (C–E).

Epithelial nerves (instead of days 7–14 in keratectomy or day 21 in perilimbal incision). Whereas the smaller wound size in this study (2 mm instead of 4 mm or more) could account for the differences in the total nerve regeneration period, wound size alone may not account for the vast differences in the time of onset of nerve regrowth. Thus the cryodamage model is a more efficient model for experimental studies. Second, in the cryodamage wound, necrotic cells and nerves were completely removed after 3 days, leaving a clear zone of undestroyed matrix materials in the stroma. Thus the repair process can be examined unambiguously by microscopy in the absence of preexisting cells and nerves. In the perilimbal incision model, initial nerve degeneration took 2 weeks before repair began, which could hamper the visual differentiation between the old and new nerves. Third, the sparing of destruction to the matrix
Fig. 6. Enface sections of cryodamage wound showing changes in the subepithelial nerve plexus (gold chloride staining). (A) Necrosis of damaged nerve plexus inside the wound is identified by aggregation of macrophage-like cells (*), exposed fibers of deteriorating nerves (small arrows), and separated Schwann cells (large arrows). Note the presence of necrotic keratocytes (arrowheads) in the stroma (day 2). (B) In the wound periphery, straight neurites (small arrows) grow from the remaining distal end of a subepithelial nerve (large arrow) (day 6). (C) Crisscross pattern of straight, long neurites (small arrows) in the subepithelial stroma. Large arrow indicates a regenerating subepithelial nerve. The wound margin is toward the upper left corner (day 10). (D) Branches of stromal nerves (large arrows) extend into the wound periphery (encircled by arrowheads) and give rise to regenerating subepithelial nerves (small arrows) (day 5). (E) Regenerating subepithelial plexus consists of proximally located medium-caliber nerve (large arrow) and interconnecting loops of small-caliber nerves (small arrows) (day 7). (F) By day 10, a complete plexus of subepithelial nerves (arrows) is regenerated in the center of the wound (encircled by arrowheads). Part of the section includes a patch of darkly stained epithelium (*). Calibration bar is 20 µm (A), 50 µm (B, C, also applies to E) or 0.5 mm (D, F).

Components permits an interpretation of nerve regrowth in the cryodamage model without a concern for possible influences from an otherwise reparative matrix environment. In this study no scar tissue was detected in the wound, which apparently is suspected of blocking stromal nerve regrowth in the perilimbal incision model. Fourth, the transcorneal freezing technique accomplishes the equivalent of nerve tran-
Fig. 7. Enface sections of cryodamage wound showing the association of Schwann cells with regenerating subepithelial nerves (gold chloride staining). (A) Newly regenerated subepithelial nerves (arrows) are bare of Schwann cells. A patch of epithelium (*) is also shown (day 4). (B) Part of a plexus (between two arrows) is covered with three medium-stained Schwann cells (arrowheads), and another part is bare (*) (day 4). (C) Three lightly stained Schwann cells (arrowheads) form a plexus-like network. A regenerating subepithelial nerve (large arrow) grows along part of the network (distal tip of the nerve is indicated by *). The tips of the dendritic processes of the Schwann cell on the right is indicated by small arrows (day 6). (D) The nerve (arrow) and branch junction (arrowhead) of a regenerated subepithelial plexus show normal close association of Schwann cells (day 6). Calibration bar is 50 \( \mu \)m (A, also applies to B–D).

section without cutting through the cornea and leading to leakage of the aqueous humor and other complications. Although the model is not directly relevant to clinical situations, its use as an experimental model should yield rich information on nerve regeneration, as this study has shown.

In the cryodamage model, three features of keratocyte repair can be recognized. First, all the necrotic keratocytes were removed within 2 days by invading macrophage-like cells. Second, mass migration of keratocytes from the wound periphery accounted for the initial repopulation (up to day 5) of keratocytes in the wounded stroma. The unique lobulated morphology of the migrating keratocytes has not been described before in other wound studies (e.g., incision wound\(^5\)). The slender lobulated processes may be related to cellular mobility in between the intact collagen lamellae. Third, after the initial repopulation of keratocytes, the cell density gradually increased to nearly normal by day 21, possibly due to cell mitosis.\(^5\) The ring of darkly stained spindle cells that appeared during this time may represent some keratocytes which were oriented perpendicularly (instead of parallel) to the enface plane (analogous to the edge-on view of normal keratocytes in a corneal cross-section). The significance of this change in orientation of some keratocytes to wound repair is not clear.

The novel sprouting of straight long neurites in the cryodamaged stroma is intriguing. The sprouting began as early as day 2, preceding by 1 day the onset of genuine nerve regrowth that reconstructed the normal pattern of stromal and subepithelial nerves. The pattern of sprouting was random and disorderly. All the neurites were very small in caliber, ran straight courses, turned abrupt angles, and remained in the same narrow enface plane, all of which features are categorically different from the normal corneal nerve
Fig. 8. Enface sections of cryodamage wound showing regeneration of epithelial nerves (gold chloride staining). (A) Parallel-running, wound-oriented epithelial neurites (arrows) in the wound periphery. The wound center is toward the upper right corner (day 2). (B) A single epithelial nerve (small arrow; initiation point is indicated by arrowhead) grows from a subepithelial nerve (large arrow). Epithelial cells (ep) are lightly stained (day 14). (C) A semi-leash nerve (small arrows) grows from a subepithelial nerve (large arrow). Note the linear pattern of the semi-leash. Part of an intraepithelial nerve is indicated by arrowhead (day 14). (D) Two semi-leashes (arrows) grow from two subepithelial nerves (arrowheads), initially toward the upper left corner, but subsequently turn toward the upper right corner. This field is located at the nasal periphery of the wound. The wound center is toward the upper right corner (day 7). (E) A fully regenerated basoepithelial leash (small arrows) grows from a subepithelial nerve (large arrow) and curves toward the wound center (upper direction). This field is located at the nasal periphery of the wound (day 7). (F) A regenerated leash with an unusual wavy pattern (small arrows) grows from a subepithelial nerve (large arrow) (day 10). Calibration is 20 μm (A, also applies to D; B, also applies to C, E, F).

pattern. The neurites not only invaded the wounded stroma, but also spread outside the wound, sometimes as far as the limbal periphery. It is likely that the neurites represent an injury-induced reaction of the corneal nerves that is distinct from the subsequent nerve regrowth. In some previous studies, similar straight neurites may have been observed also but were somewhat neglected. For example, in the study of keratotomy wound by Zander and Weddell,16 similar neurites were shown in their Figure 31 and Diagram 3. Rexed17 also observed similar neurites in her Figures 1 and 5 on the study of corneal grafts. In the
study based on heptanol-wound model by de Leeuw and Chan, similar neurites were shown in their Figures 7B and 8A. Therefore, the abnormal sprouting from the stromal and subepithelial nerves may not be unique to cryodamage wounding. The functional significance of the sprouting is not understood. The neurites persisted up to day 21 when genuine nerve regrowth had progressed. Thus the neurites may not serve as transitory innervation. The neurites were never associated with Schwann cells, but some did terminate on stationary keratocytes. It is unlikely that the neurites were related to the migratory phase of keratocyte repopulation in the wound, since the time course was different (days 2–21 versus days 1–5), and no contact with migratory keratocytes was apparent. Possibly, the neurite-keratocyte association may represent an ad hoc trophic interaction for some phase of nerve or stromal repair (such as biochemical synthesis). Further studies are necessary to clarify this phenomenon.

The unique physical features of the cryodamage wound provided an unprecedented opportunity to track the behavior of Schwann cells during nerve regrowth. Since all cells were frozen and removed from the wounded stroma, the repopulation of Schwann cells in the regenerated nerves originated from the intact nerves surrounding the wound. Schwann cells appeared in the proximal part of the small-caliber regenerated nerve branches (stromal and subepithelial) only 1–2 days after these branches were formed. There was a distinct pattern of proliferation of Schwann cells from the proximal-to-distal part of the growing nerve fibers, concomitant with the centripetal direction of nerve regrowth. However, 1–2 days after the first appearance of Schwann cells, some groups of Schwann cells in the subepithelial area appeared to form a plexus-like track preceding the growing nerve tip. Previous studies indicate that two types of interaction may occur between peripheral nerves and Schwann cells. The axonal surface may present mitogenic and differentiation signals to Schwann cells, thereby inducing the latter to proliferate, ensheathe the axon, and progress to myelination when appropriate. Conversely, Schwann cells may mediate nerve growth by forming guiding tracks, based on cell-surface contact. It appeared that in the cryodamage model, the axonal induction of Schwann-cell ensheatheent occurs initially in both stromal and subepithelial nerve regrowth, and Schwann cell guidance may contribute subsequently to the completion of the subepithelial plexus. A previous study based on perilimbal-incision wounding suggested that stromal nerve regrowth may depend on residual Schwann-cell channels in the wound. This did not happen in the cryodamage wound. Instead, an injury-induced interaction of growing axons and Schwann cells appeared to provide the impetus for regeneration of the stromal and subepithelial nerve pattern. The partial staining of Schwann cells by the gold-chloride technique enabled us to delineate their behavior during nerve repair in this study. More specific techniques such as immunocytochemistry may be necessary in future studies to characterize the molecular features of nerve-Schwann cell interaction.

It is paradoxical that in the cryodamage model, the regeneration of stromal and subepithelial nerves surpasses other previous models in kinetics, and yet the repair of epithelial nerves remains as incomplete as in other models. Although quantitation of neural density was not undertaken in this study, the stromal and subepithelial nerves were clearly close to normal in all aspects after 21 days, whereas the epithelial nerves consisted mostly of single axons, semileashes, and very few complete leashes. It is likely that the combined absence of scar tissue formation, prolonged degeneration of the damaged nerves, and complications derived from mechanical incision could facilitate the speedy repair of nerves in the stroma. In the case of epithelial nerves, the times taken for the incomplete repair are similar in cryodamage wounding (3 weeks), heptanol wounding (4 weeks), and keratectomy wounding (4 weeks). In all these wounds, the epithelium was regenerated from perilimbal cells before repair of the normal nerve pattern was initiated. Possibly, the reparative epithelium was not as inducive as the native epithelium (at development) in facilitating a normal and complete restoration of epithelial nerve growth. As a working hypothesis for the role of neuron-epithelial interaction in the development and maintenance of corneal innervation, we propose that the corneal epithelium may influence its own innervation by first secreting diffusible trophic factors to stimulate the regenerative function of neurons (via retrograde axonal transport) and provide an initial directional guidance to axons. Subsequently, direct contact interaction between the growing axons and epithelial cells may determine the final nerve pattern and density. To explain this common observation on reparative epithelium, we may propose also that the functional ability of the reparative epithelium may be deficient in either or both aspects of trophic-factor secretion and cell-nerve contact interaction. Previous evidence supports the concept of trophic factor secretion and cell-nerve contact interaction in the corneal epithelium. Further studies will be necessary to verify the concept of trophic deficiencies in the reparative epithelium.
Key words: corneal innervation, cryodamage, nerve regeneration, wound healing

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References