Scanning electron microscopy of corneal wound healing in the rabbit

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Corneal lesions 7.5 mm in diameter were made with an ocular trephine in rabbits. The time periods studied were 0, 30 min., and 1, 2, 4, 8, 16, and 24 hr. At the end of the time period, the cornea was flooded with 4% glutaraldehyde, buffered with cacodylate, pH 7.4, and kept moist until removed. It was then fixed for 24 hr. Half of the sample was dehydrated in graded alcohols, critical-point-dried, coated with gold palladium alloy, and viewed in an AMR-1000 scanning electron microscope at an accelerating voltage of 20 kv. From 0 to 4 hr, cell trauma, debris, and retraction are seen at the margin of the lesion. From 8 to 24 hr, a significant number of polymorphonuclear leukocytes are present over the total surface but in especially large numbers at the wound margin. At 16 to 24 hr, evidence of cell movement is present. Cells show ruffling membranes, a decreased number of microvilli, and a few filopodia along the advancing edge.

Key words: cornea, scanning electron microscopy, wound healing.

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canning electron microscopic studies have shown that the superficial corneal epithelium is composed of fine ridges which are very sensitive to chemical and mechanical stimuli. In pathological conditions, the surface becomes irregular, pits appear, and the formation of cytoplasmic protrusions and separation of cells are common. A study of epithelial abrasion in rabbits, using the scanning electron microscope, was reported in 1975 by Pfister. The immediate response to injury was separation and thickening of the basal and squamous epithelial cells at and near the margin of the wound. At 3 hr, polymorphonuclear leukocytes appeared, and after 15 hr, most of the epithelial cells at the wound margin were extensively flattened and showed a wide variety of surface ruffling and filopodia. Corneal re-epithelialization proceeded, with two or three cell layers moving over the basal lamina. Using the combined techniques of scanning and transmission electron microscopy, Kuwabara et al. studied the healing process after superficial linear wounds of the rabbit cornea. The initial step of healing was the sliding of the epithelial cells into the tissue defect. The basal cells remained at the
Fig. 1. Transmission electron micrograph showing the margin of the lesion at 24 hr. The lesion leaves the basement membrane (BM) intact, with scatter remains of basal cells (C) showing. (×37,500.)

Fig. 2. Scanning electron micrograph showing the margin of the lesion at time 0. The corneal surface (S) shows the typical hexagonal cells of normal epithelium. The margin of the lesion (L) shows evidence of cell trauma and retraction. (×1,000.)
original site, and mitotic activity was not seen until a few days later. The sliding cells extended fine processes into the tissue defect and eventually transformed into basal cells, with a thin basement membrane forming within a few days. There was no indication that the cut edges of the collagen fibers rejoined in superficial wounds.

The purpose of the present work was to study the regenerative processes of the corneal epithelium at designated time intervals following ocular trephine lesions in rabbits, with the use of the scanning electron microscope. These observations may aid in clarifying the sequence of events occurring in corneal wound healing. In addition, this study, using trephine lesions, may be compared with those using linear lesions or abrasive models.

Methods

Corneal lesions were made in four series of adult, New Zealand albino rabbits. The rabbits were anesthetized with pentobarbital, lidocaine was administered as a retrobulbar anesthesia, and lesions 7.5 mm. in diameter were marked centrally with an ocular trephine. The time periods studied were 0, 30 min., and 1, 2, 4, 8, 16, and 24 hr. With the use of an operating microscope, fluorescein was employed to delineate the lesion, and the epithelium was then denuded.

At the end of the designated time period, the cornea was flooded with 4% glutaraldehyde, buffered with cacodylate, pH 7.4, and kept moist until removed. The time period between flooding the cornea with fixative to its removal was 2 to 3 min. The cornea was then cut in half and placed in a vial of the same fixative for 24 hr. The half for scanning electron microscopic observations was dehydrated in graded alcohols, pinned on a cork, and critical-point-dried with liquid CO₂. Specimens were coated with gold palladium alloy and viewed in an AMR-1000 scanning electron microscope at an accelerating voltage of 20 kv. and a tilt of 10 to 20 degrees.

Results

Preliminary transmission electron microscopic observations show that our lesion leaves the basement membrane intact with scattered remains of basal cells (Fig. 1). A scanning electron micrograph of the margin of the lesion at time 0 is shown in Fig. 2. Hexagonal surface cells typical of normal epithelium are seen, and the margin of the denuded area shows evidence...
Fig. 4. Scanning electron micrograph showing the margin of the lesion (L) at 1 hr. There is continued evidence of retraction, and a few polymorphonuclear leukocytes are seen at the edge of the wound. (×800.)

Fig. 5. Scanning electron micrograph showing the margin of the lesion (L) at 2 hr. Significant retraction is evident. (×1,000.)
Fig. 6. Scanning electron micrograph of the lesion at 8 hr. A large number of polymorphonuclear leukocytes (P) are visible near the periphery of the lesion. (×400.)

Fig. 7. Scanning electron micrograph of the lesion at 8 hr. Areas of polymorphonuclear leukocytes (P) are seen enmeshed in an amorphous background, presumably tear film. (×1,000.)
Fig. 8. Transmission electron micrograph of the specimen shown in Fig. 7, confirming the presence of polymorphonuclear leukocytes (P) near the periphery of the lesion. Note the metal coating (M) over the cell. (×25,000.)

Fig. 9. Scanning electron micrograph of the lesion at 16 hr. showing evidence of cells moving into the denuded area. Cells were found to have evidence of partial separation from neighboring cells. Advancing cell edges, C-1 and C-2, show evidence of ruffling membranes and decreased number of microvilli. (×1,000.)
Fig. 10. Scanning electron micrograph of the lesion at 16 hr. showing the advancing edge of C-1 (Fig. 9) at higher magnification. (×10,000.)

Fig. 11. Scanning electron micrograph of the lesion at 16 hr. showing the advancing edge of C-2 (Fig. 9) at higher magnification. (×10,000.)
Fig. 12. Scanning electron micrograph of the lesion at 16 hr. showing evidence of cells moving into the denuded area. The advancing cell edge (C) shows evidence of ruffling membranes and decreased number of microvilli. (x10,000.)

Fig. 13. Scanning electron micrograph of the lesion at 16 hr. The advancing cell edge (C) is thin and shows a decreased number of microvilli and filopodia. (x10,000.)
of cell trauma and retraction. At 30 min. (Fig. 3) the wound margin shows some retraction at the epithelial edge. The traumatized cells and the amount of cellular debris appear to be less. At 1 hr. following injury, the wound edge is still sharp with continued evidence of retraction (Fig. 4). A few polymorphonuclear leukocytes are seen at the edge of the wound. Significant retraction is seen in many areas at 2 hr. (Fig. 5).

A large number of polymorphonuclear leukocytes are seen near the periphery of the denuded area 8 hr. following injury (Fig. 6). These appear to be coming from the tear film, as evidenced by many areas showing a mosaic pattern of cells enmeshed in an amorphous background (Fig. 7). We confirmed the fact that these are leukocytes by doing transmission electron microscopy on the same specimen that had been scanned (Fig. 8). This specimen was scanned, removed from the specimen mount, placed in propylene oxide, embedded in Spur, and sectioned for transmission electron microscopy. In view of the fact that the specimen had not been osmicated, we feel that the visualization is adequate to substantiate our observations.

Evidence of the cells moving into the demoded area was seen at 16 hr. Cells were found to show evidence of partial separation from neighboring cells (Fig. 9). Cells show evidence of ruffling membranes, a decreased number of microvilli and a few filopodia along the advancing edge (Figs. 10 to 12). The area shown in Fig. 12 suggests the migration of two layers of cells. This is seen only occasionally along the circumference of the lesion. The advancing edges of the cells appear thinned as they move over the denuded area (Fig. 13). The same cellular features are seen at 24 hr. following injury (Fig. 14) as described here for 16 hr.

**Discussion**

Light microscopic studies of corneal wound healing have been concerned primarily with keratocyte changes in the stroma of the cornea. In the rabbit, Wolter reported that the cellular pattern of the
stroma was lost and the cells lost their interconnections, becoming somewhat epitheliod in appearance. Baum reported that the aqueous humor could serve as an important source of fibroblasts 48 hr. after injury.

Transmission electron microscopic studies of corneal wound healing in rabbits have revealed cytological changes in all layers of the cornea. Jakus reported that in scarred rabbit cornea the stromal cells resembled those in embryonic chick cornea. Latessa and Ross observed that at 24 and 36 hr. after injury, the wound surface was covered completely by epithelial cells and the basal cells were in contact with randomly packed collagen fibrils. Polymorphonuclear neutrophils were noted, and the endothelial cells appeared altered. Matsuda and Smelser showed that both epithelial cells and fibroblasts participate in the healing of the anterior portions of the cornea and endothelial cells play an important role in the healing of the posterior portion of the wound.

Our scanning electron microscopic studies of corneal wound healing in the rabbit agree, in part, with the work reported by Pfister. He, too, used an epithelial lesion centrally placed with a trephine. At 15 hr. following injury, he described epithelial cells at the wound margin as extensively flattened and showing a wide variety of surface ruffling and filopodia. Our observations at 16 hr. show conditions similar to this description. We did see filopodia and a decreasing number of microvilli along the advancing edges of flattened superficial cells. We, however, never observed the two or three cell layers moving as a sheet over the basal lamina as described in his work.

The appearance of polymorphonuclear leukocytes on the surface of the denuded area has been reported in the literature. Our observations are in agreement with these reports. In view of the masses of cells enmeshed in an amorphous background, we believe these are brought into the area with the tear film.

Kuwabara et al., using the combined techniques of scanning and transmission electron microscopy, reported that wing and superficial cells, which have escaped direct mechanical damage, begin to slide into the wound in amebic fashion about 1 hr. after wounding. After 3 days, mitotic activity results in basal cells. Since a linear lesion penetrating the basal lamina was the experimental model in this study, a comparison with our work is not all that feasible. In our studies, the cells we have seen sliding into the tissue defect are primarily the superficial epithelial cells.

We believe it is worth mentioning that our observations of the centrally placed trephine lesion suggest that the healing process is not uniform about the circumference of the lesion. This may be due to the technique used in making the lesion or other factors involved in cell migration.

REFERENCES