

Endothelial F-actin Changes in the Alkali Burned Rabbit Cornea

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The healing mechanism of corneal endothelium after alkali burn was not completely understood. Rabbit cornea was burned with 1N sodium hydroxide for 1 minute. Endothelial F-actin was stained with NBD-phalloidin in regular sequence to find out the details of endothelial healing after alkali burn. F-actin was completely destroyed leaving a sharp margin against the unaffected area 1 hour after the burn. In the 3, 5 and 7 day specimens, highly active F-actin reactions were noted at the wound margin. New multiple F-actin layers, arising from the intact endothelium near the wound margin, were noted in the 9 day specimen. In the 8 $\frac{1}{2}$ month specimen, the endothelial defected area was covered by large primitive cells, each of which showed F-actin fiber bundles in the cytoplasm with a large nuclear shadow. Nearly all of the large primitive cells showed F-actin fibers arranged in shapes of cell junctions. Twelve months after the burn, endothelial defects were not found. Nearly all of the endothelial cells were normal in size and shape except for some mushroom-like projections toward the anterior chamber in some areas. Nineteen months after the burn, the endothelial cells were normal. Endothelial wound healing process can be continued even 1 year after the alkali burn in rabbit cornea.

Key Words: Alkali burn, endothelial healing, F-actin

Alkali burn in the cornea shows repeated and persistent corneal ulcer and severe inflammation, usually resulting in leukoma (Brown *et al.* 1969; Grandinger *et al.* 1969; Brown *et al.* 1970). The pathophysiology of repeated corneal ulcer was investigated for a long period. Berman *et al.* (1980, 1987) insisted that excessive plasmin production at the wound

would interfere with the re-attachment process of the epithelium by destroying the fibronectin, and the corneal ulcer would come out as a result. Chung and Fagerholm (1987a, b) reported that persistent endothelial damage would be one of the causes of the repeated corneal ulcer. Matsuda and Smelser (1973) reported that the endothelium was damaged after corneal alkali burn, although Descemet's membrane seemed to be normal with transmission electron microscopy. Still the exact pathophysiology of the repeated corneal ulcer was not found.

F-actin, a major component of the cellular cytoskeleton plays a key role in maintaining cellular structure and in healing wounds (Goddette and Frieden 1986; Gordon *et al.* 1982; Gordon 1983). Fujino and Tanishima (1987a, b) studied F-actin changes in rabbit corneal endothelium during wound healing in vivo with the nitrobenzoxadiazole(NBD)-phalloidin stain.

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They showed many fine fibers throughout the cytoplasm of cells in wound healing process. Kim *et al.* (1992) showed that even small changes of F-actin could be detected with NBD-phalloidin stain.

The purpose of this investigation was to evaluate the pattern of healing wounds in the rabbit endothelium with F-actin staining method so that the wound healing pattern of the human endothelium after alkali burn would be understood.

MATERIALS AND METHODS

Alkali burn in rabbit cornea

Forty white rabbits of both sexes, weighing 2.0~2.5 kg were anesthetized with pentobarbital i.v. and 0.5% proparacaine ophthalmic drops. In one cornea of each animal, an alkali wound was induced. A round filter paper, 1/4 inch in diameter (Schleicher & Schuell 740E, Nh.), soaked in 1N NaOH, was put centrally on the cornea for 60 seconds. After removal of the filter paper, the cornea was irrigated with balanced salt solution for 2 minutes. Tobramycin ophthalmic solution (0.3%) was applied to prevent corneal infection. Three rabbits each were sacrificed and enucleation was performed 1 hour, 1, 3, 6, 7, 9, 13 days, 1, 2, 3, and 6 months after burn. Two rabbits were sacrificed and enucleation was performed 8, 12, and 19 months after the burn.

NBD-phalloidin staining of F-actin filament

The cornea with scleral rim was fixed in a 10% neutral formalin for 90 minutes. A corneal button 8mm in diameter was trephined from the cornea. The endothelium with Descemet's membrane (including a small portion of posterior stroma) was removed from the overlying stroma and epithelium with forceps under the dissecting microscope. Eight radial incisions were made in the periphery of the sheet of endothelium plus Descemet's membrane to permit a flat preparation of the cornea.

The flat preparations of rabbit corneal endothelium were stained with 1.64×10^{-4} mol/l

NBD-phalloidin (Molecular Probes, Inc, Eugene, OR) for 30 minutes at 37°C for visualization of F-actin filaments. Following a buffer wash, tissues were mounted in buffer/glycerol (1:1) and photographed using an Olympus fluorescence microscope (BH2-RFCA) with filter BP495.

RESULTS

F-actin fibers of normal rabbit endothelium were mainly located adjacent to lateral cell membranes with circumferential bands forming hexagons. There were no dense F-actin fibers found in the cytoplasm. The F-actin fibers at the junction between cells were composed of two major bands running parallel along the periphery of the cell. There were numerous interconnecting strands joining these two bands.

F-actin picture 1 hour after the burn showed that the endothelial layer, which corresponded to the area where alkali-soaked paper had been applied, was completely destroyed leaving a sharp margin against the unaffected area. F-actin arrangements of the cells from the unapplied areas were not changed (Fig. 1).

In the day 1 specimen, condensed F-actin fibers at the wound margin were noted. The F-actin of the unaffected endothelium near the wound margin were polymorphic in figure (Fig. 2).

In the 3, 5 and 7 day specimens, highly active F-actin reactions were noted at the wound margin.

In the 9 day specimen, other new F-actin layers, arising from the intact endothelium near the wound margin, were noted (Fig. 3, A and B). These new layers showed multiple layered F-actin bundles that were directed toward the damaged area (Fig. 3, C). In some areas of the new layer, F-actin fibers were arranged in shapes of cell junctions (Fig. 3, D).

In the 1 month specimen, the size of the endothelium defected area was reduced. The regenerated endothelial cells at the defect margin were large in size and polygonal in shape.

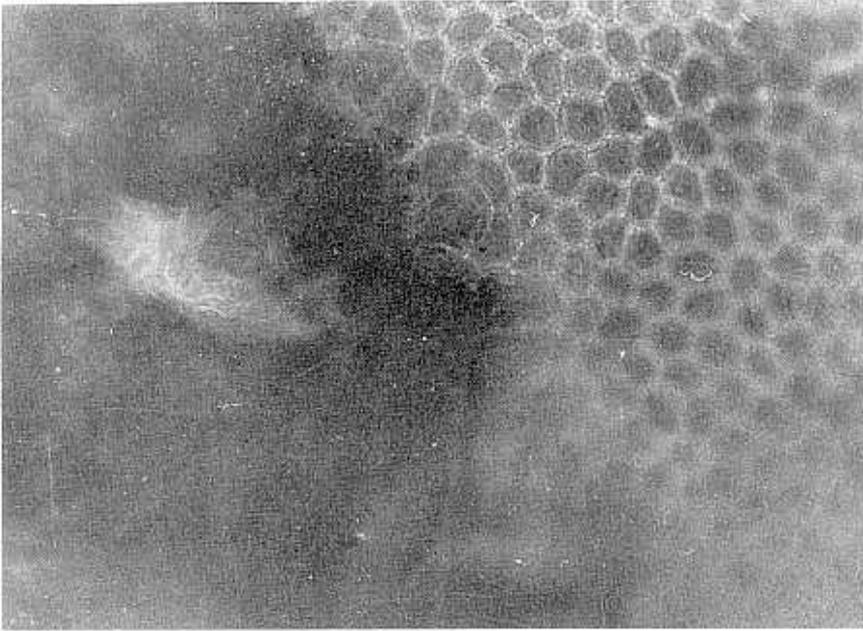


Fig. 1. NBD-phalloidin stained F-actin fibers of the rabbit corneal endothelium 1 hour after alkali burn ($\times 150$). The endothelial layer, corresponding to the area where alkali-soaked paper had been applied, was completely destroyed leaving a sharp margin against the unaffected area. F-actin arrangement of the unaffected cells were normal.

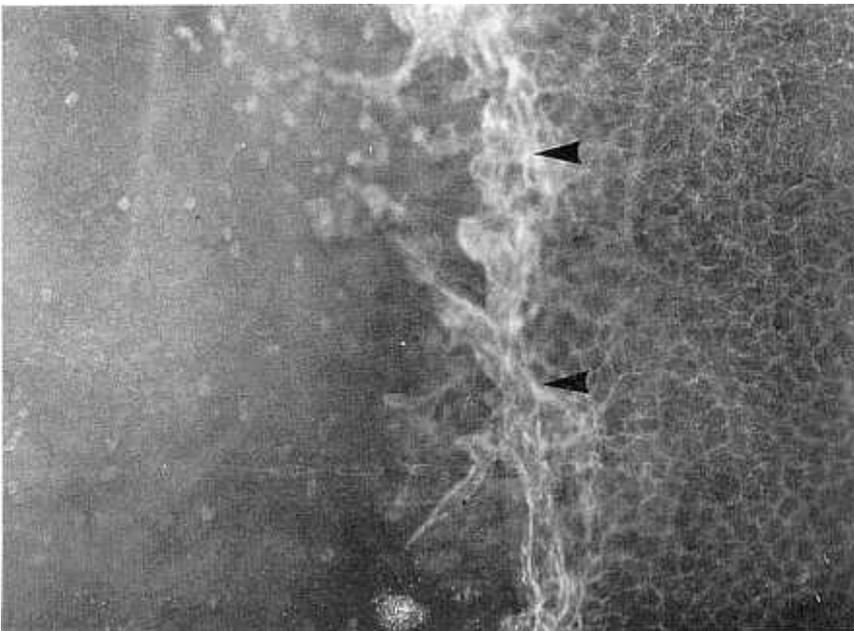


Fig. 2. NBD-phalloidin stained F-actin fibers of the rabbit corneal endothelium 1 day after alkali burn ($\times 150$). Condensed F-actin fibers at the wound margin (arrow heads) were noted. The F-actin of the unaffected endothelium near the wound margin were polymorphic in figure.

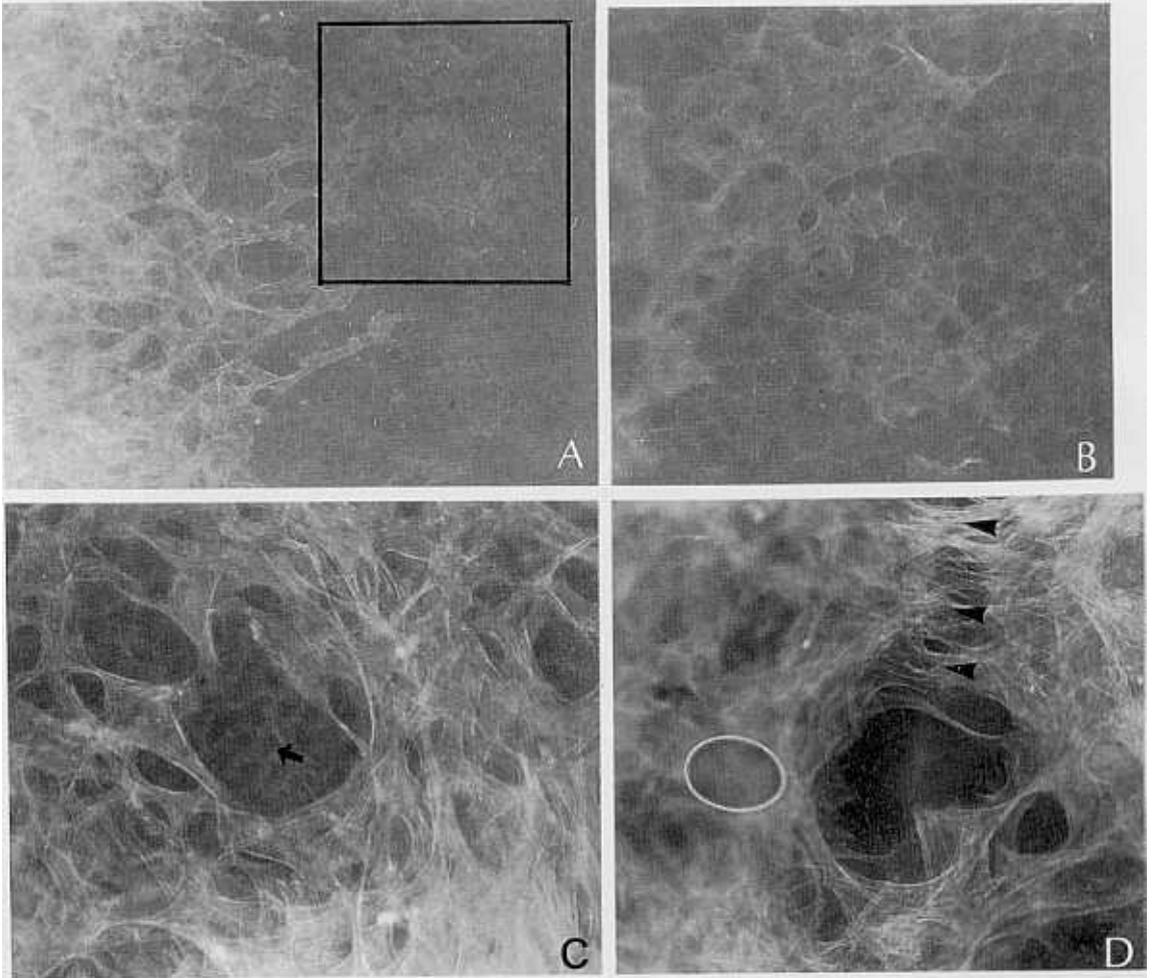


Fig. 3. NBD-phalloidin stained F-actin fibers of the rabbit corneal endothelium 9 days after alkali burn. Figure A shows that the retrocorneal membrane is arising from the normal endothelium ($\times 75$). Figure B is the higher magnified one ($\times 150$) of the marked square in figure A. Figure C shows that the retrocorneal membrane is composed of multiple layered structures ($\times 150$). Under the retrocorneal membrane, normal endothelium (arrow) was observed. Figure D shows the two cells loosely connected by cell junction structure (arrow heads) ($\times 300$).

Some of the regenerated cells at the defected area showed F-actin microspikes pointing to anterior chamber. There was no cellular F-actin at the defected area (Fig. 4).

In the 2 month specimen, the size of the endothelial defected area was reduced, but the endothelial findings at the defect margin were similar to those of the 1 month specimen.

In the 8½ month specimen, the endothelial

defected area was covered by large primitive cells (Fig. 5, A). The large primitive cells were located more basally comparing to the normally regenerating endothelium (Fig. 5, B). A large primitive cell showed F-actin fiber bundles in the cytoplasm and a large nuclear shadow in the F-actin stain. Nearly all of the large primitive cells showed F-actin fibers arranged in shapes of cell junctions (Fig. 5, C).

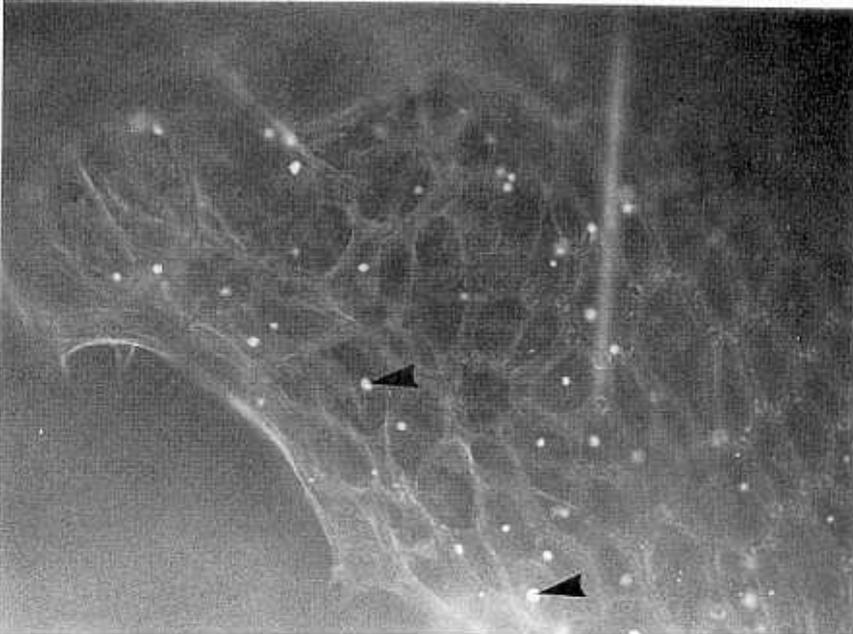


Fig. 4. NBD-phalloidin stained F-actin fibers of the rabbit corneal endothelium 1 month after alkali burn ($\times 150$). The regenerated endothelial cells at the defect margin were large in size and polygonal in shape. Some of the regenerated cells at the defect area showed F-actin microspikes (arrow heads) pointing to anterior chamber. There was no cellular F-actin at the defect area.

They were arranged in monolayer with the exception of some pilings at the cell junctions in F-actin stain. The regenerated endothelial cells showed normal size and shape. However mushroom-like projections toward the anterior chamber or F-actin spikes were sometimes noted with F-actin stain (Fig. 6, A and B). Scanning electron microscopy showed dried, white, dense material attached to the apical membrane of the regenerated endothelium (Fig. 6, C and D).

Twelve months after the burn, no endothelial defects were found. Nearly all of the endothelial cells were normal in size and shape except for some mushroom-like projections toward the anterior chamber in some areas (Fig. 7).

Nineteen months after the burn, the endothelial cells were normal and there was no mushroom like projection with F-actin stain.

DISCUSSION

The gross healing pattern of corneal endothelium was observed with both light and electron microscope (Matsuda and Smelser 1973; Kubota and Fagerholm 1991). F-actin stain enables us to observe not only the general endothelial healing pattern but also the intracellular changes (Goddette and Frieden 1986; Gordon *et al.* 1982; Gordon 1983; Kim *et al.* 1992). One minute exposure to alkali in this model showed complete destruction of the endothelium without F-actin activity. One day after burning, there were condensed F-actin fibers in the endothelial cells at the wound margin, showing endothelial regenerating activity. F-actin fibers, 3, 6 and 7 days after burning, actively appeared in a random fashion at the wound margin, suggesting active

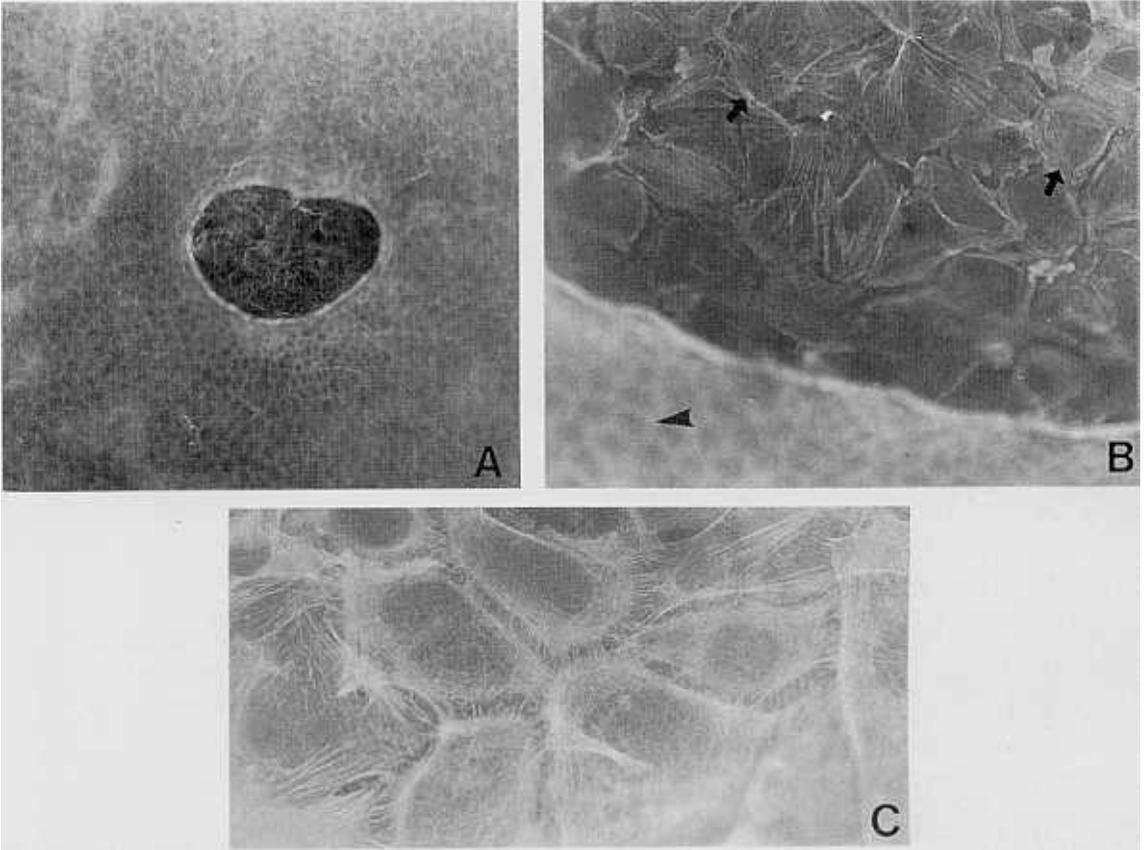


Fig. 5. NBD-phalloidin stained F-actin fibers of the rabbit corneal endothelium 8 months after alkali burn. The previously endothelial defected area is covered by large primitive cells (figure A, $\times 75$). Large primitive cells (arrows) are located more basally than the normally regenerating endothelium (arrow head) (figure B, $\times 150$). A large primitive cell shows F-actin fiber bundles in the cytoplasm and shows a large nuclear shadow. Nearly all of the large primitive cells show F-actin fibers arranged in shapes of cell junctions (figure C, $\times 300$). The primitive cells are arranged in monolayer with the exception of some piling at the cell junctions (figure C). The regenerated endothelial cells are normal in size and in shape (figure A).

wound-healing process. Nine days after the burn, several layers of amorphous structure containing F-actin fibers appeared over the normal endothelial layer. One end of the amorphous F-actin fiber layers attached to the normal endothelium layer. The amorphous layer, probably corresponding to the retrocorneal membrane mentioned by others (Matsuda and Smelser 1973; Kubota and Fagerholm 1991) sometimes showed structures resembling cell junctions. Matsuda and Smelser (1973) suggested that the cells com-

posed of retrocorneal membrane may be modified endothelial cells, not true fibroblasts. Our data also suggest that the amorphous F-actin positive layers are composed of modified endothelial cells rather than fibroblasts because the layers have direct connections to the normal endothelial layer.

One month later, defected areas of the endothelium decreased in size. The endothelial cells around the defect area were large in size. However, they remained monolayer and showed some pleomorphism and polymere-

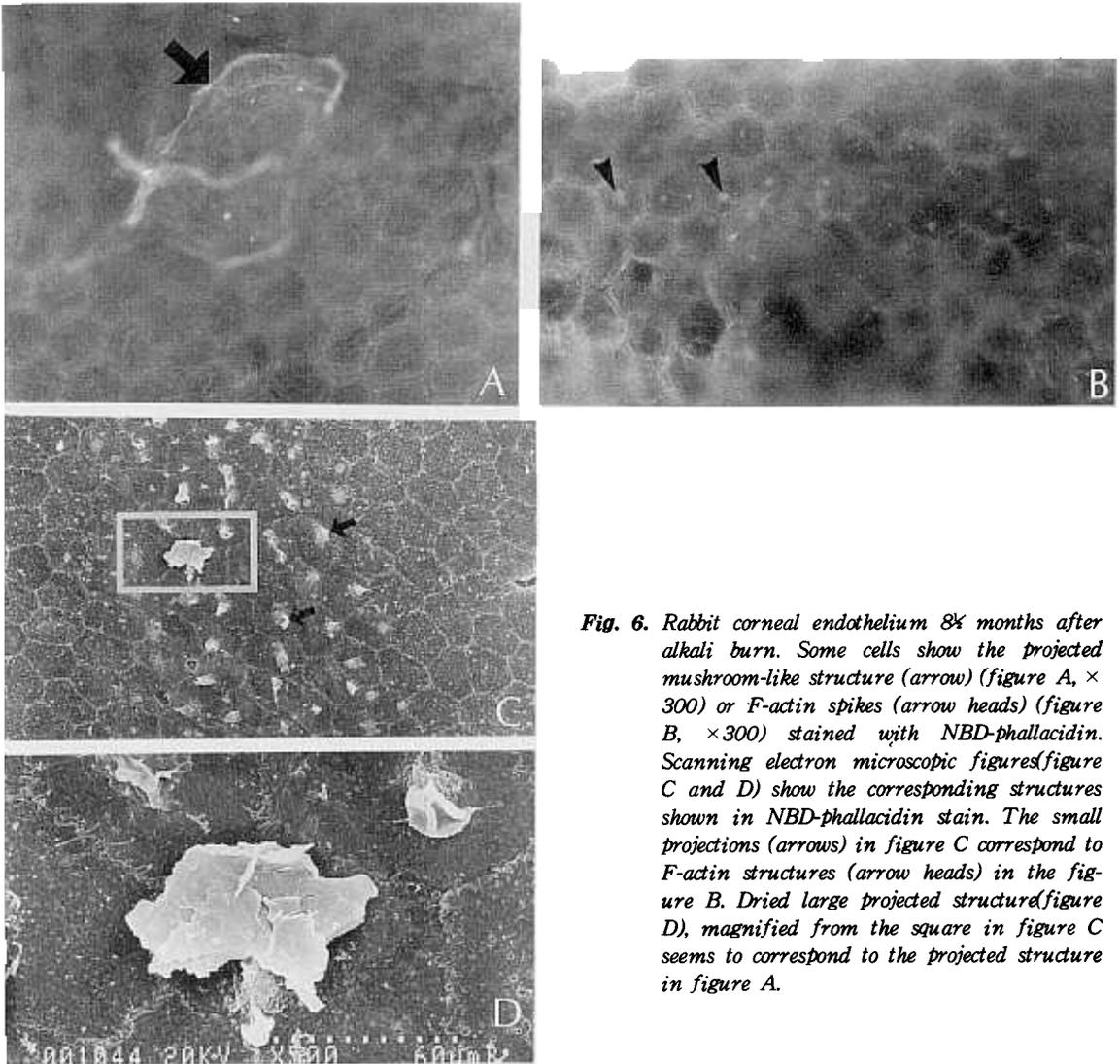


Fig. 6. Rabbit corneal endothelium 8½ months after alkali burn. Some cells show the projected mushroom-like structure (arrow) (figure A, × 300) or F-actin spikes (arrow heads) (figure B, ×300) stained with NBD-phalloidin. Scanning electron microscopic figures (figure C and D) show the corresponding structures shown in NBD-phalloidin stain. The small projections (arrows) in figure C correspond to F-actin structures (arrow heads) in the figure B. Dried large projected structure (figure D), magnified from the square in figure C seems to correspond to the projected structure in figure A.

gethism. These changes could be normally found during the endothelial healing process in the traumatized endothelial wound. The microspikes around the cells at the leading edge suggest that the cells are active in migrating (Albert *et al.* 1994).

Two month specimen showed similar findings with the 1 month specimen, except that it showed a smaller defect area compared to 1 month specimen.

In the 8½ month specimen, the previously

defected area was covered with large primitive cells. The primitive cells, arranged in monolayer, showed cell junctions between them and were located more basally compared to the normally regenerating endothelium.

Matsuda and Smelser (1973) demonstrated the retrocorneal membrane after alkali burn in the rabbit cornea with electron microscopy and named the cells composing of the retrocorneal membrane as fibroblast-like cells. They demonstrated new Descemet's mem-

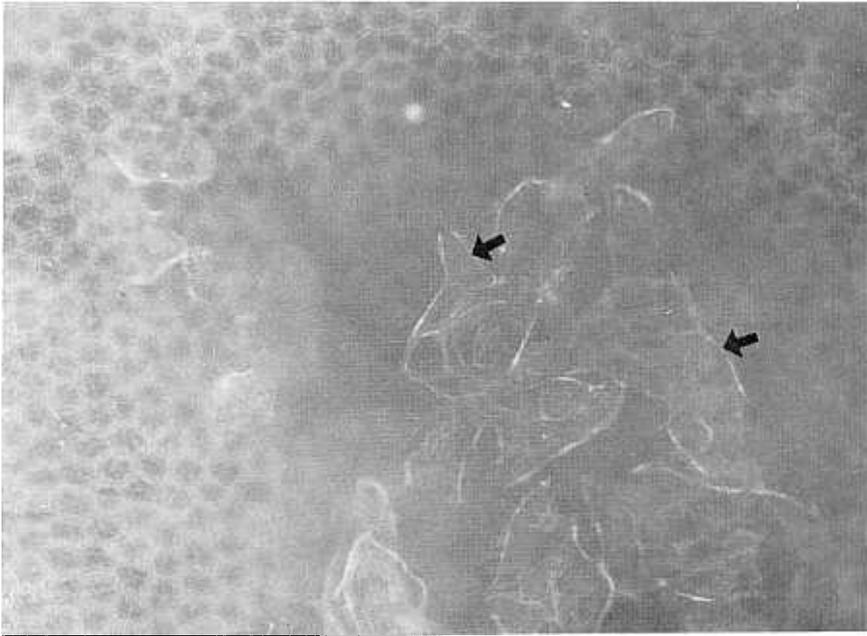


Fig. 7. NBD-phalloidin stained F-actin fibers of the rabbit corneal endothelium 1 year after alkali burn ($\times 150$). The regenerated cells were normal in shape and in size. However mushroom-like projections (arrows) toward anterior chamber were sometimes noted.

brane and new growing endothelial cells over the retrocorneal membrane. At that period, the fibroblast-like cells in the retrocorneal membrane decreased in number after covering the denuded areas and would finally convert to endothelial cells.

Kubota and Fagerholm (1991) described that a new second Descemet's membrane was formed and fibroblast-like cells were observed between the old and new Descemet's membrane. They also mentioned that the endothelium covered the new Descemet's membrane on its posterior side.

The 8½ month specimens in this study show that the primitive cells correspond to the fibroblast-like cells are those Matsuda and Smelser (1973) and Kubota and Fagerholm (1991) described. Although Matsuda and Smelser (1973) mentioned that the structure of the fibroblast-like cell was intermediate between those of fibroblast and endothelial cells. Monolayer arrangement, uniform cell junctions, and the regular shape of the cells in

these specimens were more similar with the cultured rabbit endothelium than the cultured fibroblast (Jumblatt *et al.* 1988).

One year specimens with F-actin stain showed endothelium with mushroom-like cytoplasmic projections toward the anterior chamber in some areas. Matsuda and Smelser (1973) reported that perfectly normal endothelial cells were observed 6 weeks after the alkali burn. Kubota and Fagerholm (1991) reported that the endothelium covered the new Descemet's membrane on its posterior side 6 months after the burn and that normal appearing endothelial cells covered the posterior corneal surface 12 months after the burn. The specimens in this study showed that endothelial defects remained 8½ months after the burn and showed abnormal cytoplasmic projections even 12 months after. The data differences between several authors might be from different rabbit species or different alkali burn method.

The data in this study demonstrated that

the complete endothelial healing may not occur even 12 months after the alkali burn.

When there is a large defect in the endothelial layer, it is well known that there would be epithelial defect (Joyce *et al.* 1989). The data in this study suggest that the persistent corneal ulcer after the alkali burn would be related with the persistent endothelial defects and incomplete endothelial healings.

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