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Morphological Changes in Rabbit Corneal Endothelium after Surgical Injury

Masanori Miyazaki,* Toshiro Tanaka and Teruo Nishida*

Department of Ophthalmology, Yamaguchi University School of Medicine, Ube, Japan

Purpose: To understand the responses of the corneal endothelium to different types of surgical insults, we chronologically investigated morphologic changes in rabbit endothelial cells after injury.

Methods: We performed a mechanical incision, epithelial ablation, or excimer laser irradiation on rabbit corneas and observed the changes in the endothelial cells for up to 2 weeks after surgery under a light microscope and an electron microscope.

Results: Although we observed enlargements of intercellular spaces between neighboring endothelial cells, intercellular adhesion complexes were maintained and the cells joined tightly near their apexes immediately after each procedure. We observed no signs of endothelial cell degeneration after the procedures, but we did observe many Golgi apparati, rough-surfaced endoplasmic reticula, and secreted granules, indicating that the cells had been activated. After each procedure, varying periods of time were required before the intercellular junctions and spaces returned to normal.

Conclusion: These results suggest that the different kinds of surgical injuries affected the corneal endothelium in different ways, but that the early changes were reversible. **Jpn J Ophthalmol 2000;44:342–347** © 2000 Japanese Ophthalmological Society

Key Words: Corneal endothelium, excimer laser photorefractive keratectomy, radial keratotomy.

Introduction

The corneal endothelium consists of a single layer of polygonal cells covering the posterior surface of the cornea. The cornea itself swells easily because of the water-absorbing pressure of the stroma and the intraocular pressure from the anterior chamber. Both the barrier and pump functions of the endothelial cells counteract pressure caused by swelling. The balance created by the barrier and the pump functions regulate the water content of the corneal stroma. There are many intercellular junctions among corneal endothelial cells. Among those junctional apparati are tight focal junctions at the apical surface of the endothelium. The tight apical junctions serve as a barrier, but they are not impenetrable to the passage of water. In addition, an osmotic pressure gradient is created by the active transport of intracellular ions from endothelial cells to intercellular space. A pumping mechanism also transfers water from the stroma to the anterior chamber according to the gradient. Water content in the corneal stroma is regulated by the barrier and pumping functions as well as by the cornea's natural tendency to swell. Thus, the endothelium plays a very important role in maintaining the clarity and the structure of the cornea.[1–3]

To correct refractive errors, surgery is being performed on transparent corneas of eyes that have sufficient visual acuity. The loss of corneal transparency or the loss of best-corrected visual acuity is not an acceptable postoperative outcome in this type of surgery. Basic and clinical studies have pointed out the problems of refractive surgery on the cornea.[4–8] Because corneal endothelial cells show little proliferative activity after birth, particularly in the human

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*Current affiliation: Department of Ophthalmology, Faculty of Medicine, Kyushu University School of Medicine, Kyushu, Japan

Correspondence and reprint requests to: Teruo NISHIDA, MD, DSc, Department of Ophthalmology, Yamaguchi University School of Medicine, 1-1-1 Minami-Kogushi, Ube-shi, Yamaguchi-ken 755-8505, Japan

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eye, understanding the responses of endothelial cells to surgical insults is very important, not only for the outcome of refractive surgery, but also for postoperative management of the cornea.

In order to understand endothelial cell responses to various types of surgical insults to the cornea, we performed three types of surgery—incision, epithelial ablation, and excimer laser irradiation—on rabbit corneas, and we chronologically observed early changes in the morphology of endothelial cells using light and electron microscopy.

Materials and Methods

Thirty-two Japanese white rabbits (male, 1.5 kg; KBT Oriental, Tosu City, Saga) underwent general anesthesia by administration of ketamine hydrochloride (Ketalar® 50; Sankyo Pharmaceutical, Tokyo) and xylazine hydrochloride (Seractar®; 2% injection, Bayer, Tokyo), and local anesthesia by eye drops of 4% lidocaine. The rabbits were divided into three groups and underwent (1) corneal incision, or (2) epithelial ablation, or (3) excimer laser irradiation. (1) Using a diamond knife, an incision 6 mm long and 250 μm deep was made in the center of the cornea. (2) After the center of the cornea was marked with a 6-mm trephine, the epithelium was mechanically ablated with a spatula in this area. (3) After mechanical epithelial ablation, irradiation with an excimer laser was performed on an area 6 mm in diameter, and the stroma was resected using an excimer laser-generating apparatus (EC-5000; Nidek, Gamagori) at photorefractive keratectomy (PRK) mode corresponding to 3D of the apparatus.

At 6 hours, 1 day, 3 days, 1 week, and 2 weeks after each surgery, animals were euthanized by injection of pentobarbital sodium (Nembutal®; Dainippon Pharmaceutical, Osaka) into an ear vein. The rabbits were immediately perfused with 2% glutaraldehyde. The eyeballs were enucleated and fixed with 2% glutaraldehyde and 2% osmium tetroxide. After dehydration with acetone, specimens were embedded in epoxy resin. Sections for light microscopy were prepared and subjected to histological examination under a light microscope (Axioskop 50; Zeiss, Oberkochen, Germany) after staining with toluidine blue. Ultra-thin sections were stained with uranyl acetate and lead nitrate and the ultrastructure was examined with a transmission electron microscope (JOEL-200X; Nippon Denshi, Tokyo).

Care and treatment of animals conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Results

Corneal Incision

We opened the site of the incision in a V-shape 6 hours after surgery. Using a light microscope, we were able to observe some migration of the epithelium toward the injured area. The corneal stroma was swollen and thickened by permeation of water from the tear fluid. Gaps were found in the endothelial layer under the incised site (Figure 1a). Using electron microscopy, we located these gaps at the intercellular space between the endothelial cells. Several intracellular vacuolations were detected in mitochondria. The structure of the intracellular organellae was similar to that of organellae in the intact cornea. Tight apical junctions were well maintained (Figure 1b). No significant changes were observed in the rest of the endothelium. After 1 day, the incised site had been resurfaced by epithelial cells, and the stromal edema had subsided. Increases of rough-surfaced endoplasmic reticula were observed, indicating that the endothelial cells had been activated. However, the intercellular spaces had disappeared (Figures 1c, d). After 3 days, 1 week, and 2 weeks, no significant changes were observed in the endothelial cell layer. The structure of the intracellular organellae had returned to that of organellae in the intact cornea.

Epithelial Ablation

Six hours after epithelial ablation, light microscopy did not show resurfacing of the defects. The stroma at the ablated region was edematous because of the lack of epithelial cells. Gaps were observed in the endothelial layer just under the region of epithelial ablation (Figure 2a). Under examination with an electron microscope, these gaps appeared as increased intercellular spaces between endothelial cells like the intercellular spaces observed after corneal incision. Intracellular vacuolations were remarkably observed in mitochondria. Increased numbers of Golgi apparati and secretory granules, and the partial elongation of rough-surfaced endoplasmic reticula showed that the cells were activated (Figure 2b). By the third day, the ablated region had been resurfaced by the migration of epithelial cells, and we observed multiple layers of epithelial cells. These findings indicate the progress of epithelial repair. The intercellular space (gaps) between endothelial cells had decreased somewhat. After 1 week, the epithelial cells showed further differentiation, and the shape of the endothelial cells had been restored to the morphology of normal corneas observed by electron microscopy (Figures 2c, d). After 2 weeks, no further

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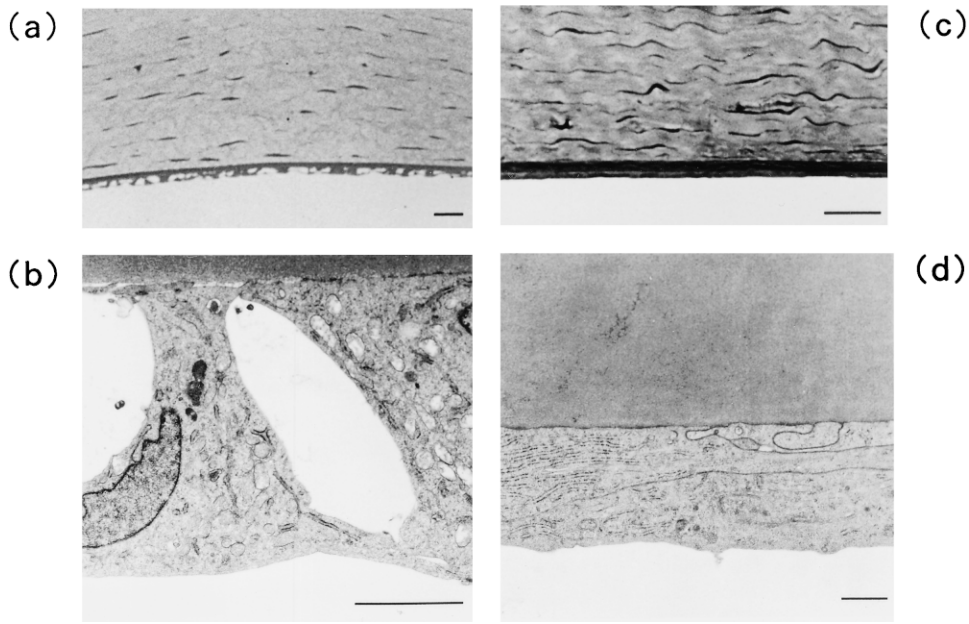

Figure 1. Morphological changes after corneal incision. (a) Light micrograph 6 hours after surgery (toluidine blue stain). Vacuoles are found in endothelial cell layer just under incision site. Bar = 50 μm. (b) Transmission electron micrograph 6 hours after surgery. Intercellular spaces in endothelial cell layer just under incision site are enlarged. Although many Golgi apparati, rough-surfaced endoplasmic reticula, and secretory granules are found in cytoplasm, and cell activation is observed, no detachment of tight junction in apex in anterior chamber side is found. Bar = 1 μm. (c) Light micrograph 1 day after surgery. Vacuoles in endothelial cell layer have disappeared, and morphology similar to that of normal corneal endothelial cells is observed. Bar = 50 μm. (d) Transmission electron micrograph 1 day after corneal incision. Structure of organellae in endothelial cells is normalized, intercellular spaces among endothelial cells have disappeared, and morphology similar to that of normal endothelial cells is observed. Bar = 1 μm.

changes in the morphology of the endothelial cells were observed.

Excimer Laser Irradiation

Six hours after irradiation, epithelial resurfacing was not completed. The tight apical junction of the endothelial cells was maintained at the irradiated site, and intercellular spaces between endothelial cells were like those observed after corneal incision and epithelial ablation The appearance of many Golgi apparati, rough-surfaced endoplasmic reticula, and secretory granules indicated that the endothelial cells had been activated. Irregular interface between corneal endothelium and Descemet's membrane was observed (Figures 3a, b). On the third day, resurfacing of the epithelial defect was completed, but intercellular spaces (gaps) remained in the endothelium. After 1 week, epithelial cells had proliferated and showed differentiation. However, spaces still remained among the endothelial cells. This result was quite different from results observed after epithelial ablation. Two weeks after irradiation, no gaps were found in the endothelial cell layer, and the morphology had been restored to that seen in normal cornea by electron microscopy (Figures 3c, d).

Discussion

In the present study, the early responses of corneal endothelial cells to three types of surgery—corneal incision, epithelial ablation and excimer laser irradiation—were morphologically investigated. Formation of intercellular spaces among corneal endothelial cells was observed shortly after the surgery. Although the recovery speed differed for each surgery type, the endothelial gaps disappeared as the epithelial defects were resurfaced. These results suggest that the early responses of the corneal endothelium were morphologically reversible.

The cornea swells easily due to the water-absorbing property of the stroma and water-permeating pressure due to the intraocular pressure. Although the mechanism by which the cornea maintains its thickness is not yet fully understood, the pump-leak theory of Maurice[1] has been widely accepted. Intercellular spaces between corneal endothelial cells are 25–45 nm. In some areas, narrow gaps of about 3 nm

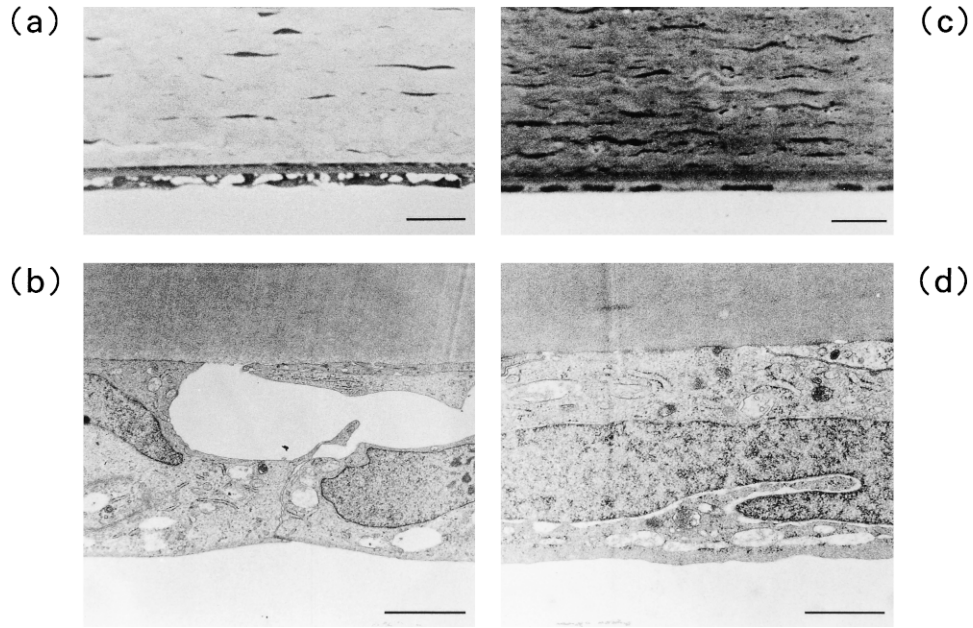

Figure 2. Morphological changes after epithelial ablation. (a) Light micrograph after 6 hours (toluidine blue stain). Vacuoles are found in endothelial cell layer just under epithelial ablation site. Bar = 50 μm. (b) Transmission electron micrograph after 6 hours. Intercellular spaces are seen in endothelial cell layer just under epithelial ablation site. Many Golgi apparati, rough-surfaced endoplasmic reticula and secretory granules are found in cytoplasm, and cell activation is observed. Bar = 1 μm. (c) Light micrograph after 1 week. Vacuoles in endothelial cell layer have disappeared, and morphology similar to that of normal corneal endothelial cells is observed. Bar = 50 μm. (d) Transmission electron micrograph 1 week after epithelial ablation. Structure of organellae in endothelial cells is normalized, intercellular spaces among endothelial cells have disappeared, and morphology similar to that of normal endothelial cells is observed. Bar = 1 μm.

were present at the apical surface of the endothelium. By contrast, the apex of the anterior chamber side has tight focal junctions with smaller spaces that are different from the spaces in the corneal epithelium. Despite its tightness, this type of junction allows some water to pass through.[2] It is known that each endothelial cell has many Na-K pumps in its cell membrane for functions such as increasing Na concentration in intercellular space by active transport, expelling water absorbed by the stroma into intercellular space via an osmotic pressure gradient, and excretion of water into the anterior chamber.[3] Because of these barrier and pump functions, water is retained in the cornea, and the thickness and clarity of the corneal stroma are maintained. Generally, when the corneal epithelium is injured, the influx of water from the tear fluid increases and stromal edema is induced, even if endothelial functions are normal. It can also be true that when the endothelium is damaged, or if the pump function is reduced because of inflammation in the anterior chamber, water excretion is disturbed, and stromal edema is induced, although there is no water influx from the tear fluid.

We previously reported the histopathological and immunohistochemical studies in corneal epithelium and stroma after corneal surgery.[9] In the present study, we focused on the endothelial changes after corneal surgery. Table 1 summarizes the morphological changes in the corneal epithelium and endothelium in response to the three types of surgery, corneal incision, epithelial ablation and excimer laser irradiation. After a corneal incision, intercellular gaps between endothelial cells disappeared and epithelial defects were completely resurfaced. After epithelial ablation and excimer laser irradiation, there was a lag between epithelial resurfacing and morphological recovery of the endothelium. The time required for the recovery of endothelial morphology was longer after excimer laser irradiation than after epithelial ablation. The probable reason for the delay in recovery is that excimer laser irradiation was not limited to the epithelium but extended to the stroma as well, so that a longer period was required for complete endothelial repair. After three types of surgery, there were many Golgi apparati, rough-surfaced endoplasmic reticula and secretory granules in the cytoplasm, suggesting activation of the endothelial cells. No intracellular vacu-

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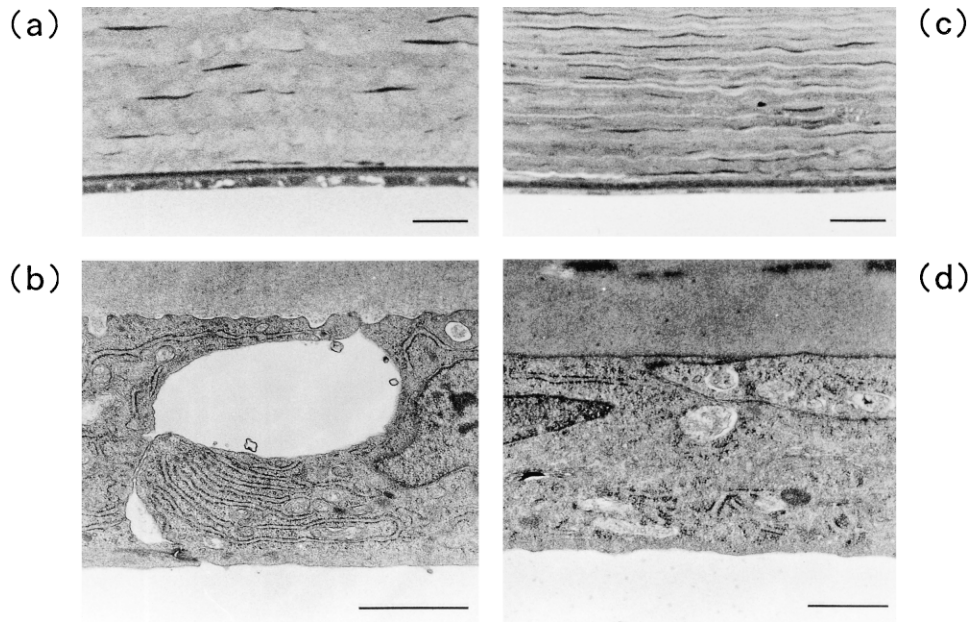

Figure 3. Morphological changes after excimer laser irradiation. (a) Light micrograph after 6 hours (toluidine blue stain). Vacuoles are found in endothelial cell layer just under irradiation site. Bar = 50 μm. (b) Transmission electron micrograph after 6 hours. Intercellular spaces in endothelial cell layer just under irradiation site are found, but tight apical junction is not detached. Many Golgi apparati, rough-surfaced endoplasmic reticula and secretory granules are found in cytoplasm, and cell activation is observed. Bar = 1 μm. (c) Light micrograph after 2 weeks. Vacuoles in endothelial cell layer have disappeared, and morphology similar to that of normal corneal endothelial cells is observed. Bar = 50 μm. (d) Transmission electron micrograph after 2 weeks. Structure of organellae in endothelial cells is normalized, intercellular spaces among endothelial cells disappear, and morphology similar to that of normal endothelial cells is observed. Bar = 1 μm.

oles were found, suggesting that the endothelial cells had not degenerated. The morphology of all areas affected by the three types of surgery had resumed the appearance of an intact cornea within 2 weeks. The intercellular gaps in the endothelium disappeared as the epithelial defects were resurfaced. Therefore, the early changes in the endothelium could be considered reversible. All three types of surgical insults created intercellular gaps. The formation of the intercellular gaps seems to be a result of tight apical junctions and an increased influx of water from the tear fluid, creating a level of stromal water beyond the pumping capacity of the endothelial cells. However, there was no morphological evidence of detachment of the tight junction in the apex of the anterior chamber. Because the barrier and pump functions of endothelial cells were not destroyed, the endothelial cell layer seemed to be morphologically normal when the water permeating the stroma decreased after the endothelium had been repaired.

Recently, refractive surgery techniques such as radial keratotomy (RK), photorefractive keratectomy (PRK) using excimer laser, and laser-assisted in situ keratomileusis (LASIK) have been developed to correct refractive errors. Their indications and complications as well as clinical usefulness have been reported.[4–8] With RK, radial incision to the cornea steepens the periphery of the cornea, but the central optic area of the cornea becomes flattened and results in decreased corneal refraction. By contrast, in PRK and LASIK, part of the corneal stroma is removed with an excimer laser, and curvature of the corneal anterior surface is re-formed to modify the corneal

Table 1. Time Required for Resurfacing Corneal Epithelium and Morphological Changes of Endothelium After Various Types of Corneal Invasions

	Corneal Incision		Epithelial Ablation		Irradiation	
Time	ER	EC	ER	EC	ER	EC
6 hours	−	+	−	+	−	+
1 day	+	+	−	+	−	+
3 days	+	−	+	+	+	+
1 week	+	−	+	−	+	+
2 weeks	+	−	+	−	+	−

ER: Epithelial resurfacing; +: resurfaced, −: not resurfaced; EC: endothelial changes, +: morphological change positive, −: no morphological changes.

refraction. Regardless of the technique, the fundamental requirements for refractive surgery are an accurate achievement of expected postoperative refraction and prevention of postoperative complications. Both the accuracy and safety of the surgical outcome depend on the healing response of the cornea after surgery. Because human corneal endothelial cells do not seem to be able to proliferate, any adverse effects on the endothelial cells might lead to permanent damage to the cornea. Therefore, it seems important to examine the endothelial responses to various types of refractive surgery. As a result of RK, polymorphism and swelling of endothelial cells at the incised area[4] and decreased numbers of endothelial cells[5] have been reported. With PRK, the generation of a substance with an extremely high number of electrons has been reported in Descemet's membrane of rabbit eyes.[6–8] When excimer ablation was as close as 40 μm from Descemet's membrane, corneal endothelial cells at the irradiated site were lost.[10]

In the present study, we examined the response of corneal endothelial cells to various types of surgery and demonstrated that morphological changes shortly after insults were reversible and caused no permanent pathology. The present study, however, was performed on rabbit cornea, which has strong powers of self-reconstruction. Therefore, these results cannot be extrapolated to the human cornea. The response of the corneal endothelium to invasion should be carefully observed for an extended period.

It is evident that refractive surgery will become part of ophthalmological surgery. Although Sato's[11] pioneering anterior-posterior RK was found to induce bullous keratopathy many years after the initial surgery,[12] his work increased our understanding of the physiology of the endothelium. Because the cornea is avascular tissue, we need to know how avascular tissue heals. Most of the studies on wound-healing mechanisms have been performed on vascularized tissue. The development of refractive surgery might increase our understanding of the corneal wound-healing process. However, knowing the factor(s) that modulates epithelial and/or stromal wound healing of the cornea will improve the surgical outcome of refractive surgery and will be indispensable for developing safer and more effective techniques.

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