Confocal Microscopy of Corneal Wound Healing After Deep Lamellar Keratoplasty in Rabbits

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Objective: To compare wound healing and morphologic characteristics of the host-donor interface in rabbit corneas after maximum-depth and near–Descemet membrane anterior lamellar keratoplasty.

Design: Descriptive analysis of confocal microscopy images after 2 types of deep lamellar keratoplasty (deep stromal dissection vs total stromal resection).

Methods: Deep anterior lamellar keratoplasty (DALK) was performed in 16 rabbit eyes, with exposure of the Descemet membrane in 8 eyes (deep group) and deep stromal dissection to near the Descemet membrane in 8 eyes (near group). A full-thickness graft devoid of endothelium and Descemet membrane was sutured in place. Confocal examination of lamellar interface and wound edge was performed throughout 6 months.

Results: Four days postoperatively, confocal microscopy revealed numerous highly reflective keratocytes at and adjacent to the interface in all eyes, fewer in the deep than the near group. Keratocyte density and reflectivity returned to normal at 4 to 6 weeks (deep) and 8 to 10 weeks (near) postoperatively.

Conclusions: In the deep group, the smooth interface showed less scarring. In the near group, stroma-to-stroma healing stimulated more activated keratocytes and hence more haze. Successful DALK requires minimal central healing for clarity but significant suture-stimulated healing at the edge to prevent corneal bulge.

Clinical Relevance: Deep anterior lamellar keratoplasty is rarely accompanied by rejection, avoids entrance into the anterior chamber, and can be performed with tissue that does not have living keratocytes. Interface healing is a determinant of the final visual acuity; depth of the lamellar bed is a major determinant of the healing response. Although dissection to bare the Descemet membrane is more difficult, there is less keratocyte activation and scarring.


Deep anterior lamellar keratoplasty (DALK) is a surgical procedure performed mainly to treat corneal scarring, keratoconus, or corneal thinning with an intact corneal endothelium. During maximum (total) DALK, the whole corneal stroma of the host is removed to expose the smooth surface of the Descemet membrane. The donor corneal button, free from the endothelium and Descemet membrane, is then sutured onto the recipient eye.

The advantages of DALK are its very low incidence of graft rejection, because the endothelium is not transplanted, and avoidance of the decrease in endothelial cell numbers seen after penetrating keratoplasty. There is less risk of intraoperative complications because DALK does not require entry into the globe.

Confocal microscopy can be used to follow the healing process after corneal lamellar surgery. Quiescent keratocyte nuclei normally appear in confocal microscopy as bright oval or bean-shaped objects against a dark background. Cellular processes are not evident in the resting state. The proliferating, activated repair fibrocyte or myofibroblast has a prominent, rough endoplasmic reticulum that is not seen in the quiescent keratocyte, implying active protein synthesis. Consequently, the activated keratocyte has greater corneal light backscattering than the quiescent keratocyte during confocal microscopy. As a result, activated keratocytes have a large, highly reflective appearance in confocal images, and their cytoplasmic processes are often visible.

Although DALK is clearly defined by baring of the Descemet membrane, the operation sometimes does not achieve this goal, resulting in a thin layer of deepest stroma remaining attached to the Descemet membrane. We sought to compare...
healing in terms of the appearance and disappearance of activated keratocytes after the ideal DALK (having the completely bare Descemet membrane [the deep group]) with eyes in which we intentionally left a part of the deep stroma attached to the Descemet membrane (the near group) using confocal microscopy.

**METHODS**

**SURGICAL PROCEDURE**

Sixteen New Zealand white rabbits weighing 3 to 4 kg were used as recipients (1 eye each), and both eyes of 8 New Zealand white rabbits weighing 3 to 4 kg were used as donor eyes. No eyes showed any corneal opacity, and no imperfections were found on the corneal surface based on detailed macroscopic and slitlamp examination. All animals were treated in accordance with the guidelines of the Association for Research in Vision and Ophthalmology for the Use and Care of Animals in Research.

The operations were performed under general anesthesia consisting of an intramuscular injection of xylazine hydrochloride (5-10 mg/kg) to produce a state of sedation with a shorter period of analgesia and ketamine hydrochloride (35-50 mg/kg) and topical proparacaine hydrochloride anesthesia. After complete draping of the rabbit, an eyelid speculum was placed into the right eye. Usual asepsis and antisepsis techniques using an iodine cleaning solution were used.

To perform DALK, an 8-mm Barron disposable vacuum trephine was used to make an approximately 75% to 80% thickness cut into the stroma. Each quarter turn of the plastic spokes of the trephine cuts about 0.15 mm of the corneal thickness, so we made about 5 quarter turns to cut approximately 0.75 mm, i.e., 75% to 80% of the peripheral corneal thickness. A sterile 30-gauge needle was attached to a 1-mL air-filled syringe. The tip was inserted bevel down into the corneal stroma deep into the trephination groove. When the tip of the needle was in the desired location, the plunger of the air-filled syringe was depressed until a large bubble was noted. Using a disposable disc knife, we performed dissection layer by layer starting at the trephination edge and guided by opacified whitish stroma until we reached the delicate, shiny, and smooth Descemet membrane in the deep group or to the clear, fine, deep stromal layer adjacent to the Descemet membrane in the near group.

The donor corneoscleral rim was then trephined from the endothelial side to the desired diameter by means of a Barron corneal punch. Donor grafts were made 0.25 to 0.50 mm larger than the recipient’s bed to avoid tension on the wound during healing and to decrease postoperative astigmatism and folds in the Descemet membrane. The donor button was then mounted epithelial side down on a sterile plastic support. The Descemet membrane and endothelium were then removed manually from the donor button. This maneuver was facilitated by using 0.8 mg of trypan blue dye. Before applying the dye, the posterior surface of the donor button was gently touched with a cellulose sponge to damage or remove the donor endothelium. Then the dye was applied onto the button to stain the Descemet membrane, and the posterior surface of the button was gently swabbed with a dry Weck cell sponge or grasped by 0.12-mm or toothed Colibri forceps to completely remove the blue-stained Descemet membrane and the endothelium. If the endothelial side of the donor cornea is wiped with a cotton swab or wick sponge, the Descemet membrane can be removed easily, leaving a completely smooth surface on the stromal side. The donor button, free from the Descemet membrane and endothelium, was then transferred to the recipient bed by means of a Paton spatula and sutured to the recipient bed with 12 interrupted 10-0 nylon sutures with buried knots.

The mean time for each procedure was 40 minutes (standard deviation, 10 minutes). All eyes were treated with prednisolone acetate, 1%, and moxifloxacin eye drops 4 times a day for 1 week, and all rabbits received 0.05-mg/kg buprenorphine (analgesic) intramuscularly for 2 days.

**SLITLAMP AND IN VIVO CONFOCAL MICROSCOPY**

All rabbits were subjected to routine slitlamp examination postoperatively at 1 day, 4 days, and 1 week; weekly for 1 month; and monthly for 6 months. In vivo confocal microscopy was performed on the corneas of all rabbits postoperatively at 4 days and 1 week, then weekly for 1 month, and monthly for 6 months using tandem scanning confocal microscopy. Corneas of the preoperated eyes were scanned with the same microscope to differentiate between unoperated corneas under a resting state with quiescent keratocytes and operated corneas with activated keratocytes postoperatively. Tandem scanning confocal microscopy uses a scanning confocal microscope with a Nipkow disc with 40-µm pores, which allows a 2-dimensional resolution of 1.3 to 2.0 µm and a vertical resolution of 12 µm. The confocal microscope had a modified speculum objective lens. Light is provided by a remote mercury or xenon lamp. Thus, the microscope and the subject are isolated from the heat and vibration produced by the lamp assembly. This arrangement provides optically sectioned in vivo images with ×230 magnification and high resolution. Video sequences were reviewed at least twice and evaluated in a masked fashion. The microscope had an objective lens with an aperture of ×24.0/0.60, a concave surface, and a working distance of 0 to 1.5 mm.

In the current study, evaluation of active keratocytes was done in a masked fashion; the differences were documented and very clear. Confocal pictures were analyzed in a masked fashion, often by 2 observers, who agreed completely over the pictures.

Under general anesthesia, the rabbit was placed in a supine position in a custom-made resin holder. The microscope objective lens was disinfected with isopropyl alcohol, 70%, before and after the examination. Methylcellulose was used as an optical coupler between the cornea and the tip of the water-immersion objective. The lens objective was manually advanced until the medium was in contact with the central cornea. A series of confocal images was recorded as the focal plane was advanced manually or automatically from the epithelium posterior to the endothelium. The position of the optical sec-

![Figure 1. Confocal micrograph showing a randomly arranged extracellular matrix (arrows). The image was taken 7 days after deep anterior lamellar keratoplasty in which the deep stroma was left attached to the Descemet membrane. Scale bar measures approximately 100 µm.](image-url)
tion could be advanced or retracted by an internal lens without changing the position of the front surface of the objective. Images were displayed in real time on a monitor and recorded by a super-VHS recorder through a charge-coupled device video camera onto digital videotape for later playback and analysis.

RESULTS

IN VIVO CONFOCAL MICROSCOPY

In all eyes, the epithelium appeared normal. Preoperated corneas exhibited quiescent keratocytes. Quiescent keratocyte nuclei appeared through confocal microscopy as bright, oval or bean-shaped objects against a dark background. Cellular processes were not evident in this resting state.

Four days postoperatively, all operated eyes of both groups had an apparent increase in the density of large, highly reflective keratocyte nuclei in the surgical interface and adjacent to it; the bright keratocytes were associated with prominent interconnecting cell processes. Activated keratocytes or repair fibrocytes lay down repair extracellular matrix (Figure 1). In the deep group, the density, brightness, and reflectivity of activated keratocytes at the interface were less compared with that in the near group.

The numbers of activated keratocytes peaked at 1 week in all operated eyes of both groups. Some of the highly reflective keratocytes appeared considerably larger than others. After 4 to 6 weeks in the deep group and 8 to 10 weeks in the near group, keratocyte morphology, density, and reflectivity had returned to normal, and large, bright, activated keratocyte nuclei were no longer seen (Figure 2 and Figure 3).

In both groups, the interface was easily identified in 25% of the cases, because numerous highly reflective interface particles were present, which may represent foreign bodies, debris, or inflammatory cells. Some of these particles were transient in nature (Figure 4). The interface was also identified by the presence of numerous highly reflective keratocytes and their cell processes, repair extracellular matrix, and the presence of homogeneous reflectivity.

Four days postoperatively, no keratocytes were found adjacent to the wound edge in either the deep or near groups (Figure 5A). Seven days after wounding, apparent keratocytes surrounding the area of cell loss began to repopulate the wound area, and many activated keratocytes were found beneath the cut edge aggregating into a dense cluster mainly around the sutures (Figure 5B). In both groups, the more anteriorly located keratocytes remained quiescent at all times.

INTERFACE HAZE

Clinical stromal haze 2 to 3 weeks postoperatively in the deep group was subjectively graded as trace (defined as haze seen only with broad-beam illumination), but at 4 to 6 weeks the interface was clear. In the near group, trace interface haze was still seen 8 to 10 weeks postoperatively and remained at this level until the end of the 6-month follow-up period.

COMMENT

The process of repair in the corneal stroma begins with the activation or transformation of quiescent keratocytes into repair fibrocytes adjacent to the acellular zone. Histologically, the first detectable morphologic changes associated with an actively synthetic repair fibrocyte are an increase in cell size and an increase in the size and
number of nucleoli, many rough endoplasmic reticula, mitochondria, free ribosomes, and Golgi apparatuses.\textsuperscript{10,14} With the confocal microscope, activated keratocytes or repair fibrocytes are involved in the production of the repair extracellular matrix.\textsuperscript{18,19} Activated keratocytes were no longer visible in any corneas by 4 to 6 weeks after surgery in the deep group and 8 to 10 weeks after surgery in the near group.

In the first phase of wound repair and 4 days postoperatively, keratocytes disappeared in the area adjacent to the wound edge in both the deep and near groups. In the second phase of wound repair and 7 days after wounding, keratocytes surrounding the area of cell loss began to repopulate the wound area. It is unclear whether keratocytes surrounding the area of cell loss divide to replenish their numbers or whether new cells migrate to the wound edge. Whatever their source, these replacement cells would have been activated to become repair fibroblasts or myofibroblasts. The mechanism by which keratocytes initially disappear is not yet fully understood, though the fact that keratocytes disappear very quickly may indicate a mechanism of self-destruction, ie, apoptosis (programmed cell death).\textsuperscript{20}

We attributed postoperative stromal haze (backscattered light) in the deep group to alterations in the repair extracellular matrix; reflective, brightly activated keratocytes; and reflective interface fluid (interface edema), evidenced by the progressive regaining of corneal transparency coupled with a progressive decrease in inter-

![Figure 3](image1)

**Figure 3.** Confocal images of the interface of an eye after deep lamellar keratoplasty in which the deep stroma was left attached to the Descemet membrane. A, At 4 days, increased density, reflectivity, and brightness of activated keratocytes (white arrow) with prominent cell processes (black arrow) are demonstrated. B, At 1 month, increased density of highly reflective and bright activated keratocytes are seen. C, At 3 months, decreased haze density and quiescent keratocytes are noted. D, At 6 months, trace haze and quiescent keratocytes are visible. Scale bar measures approximately 100 µm.

![Figure 4](image2)

**Figure 4.** Two weeks after deep lamellar keratoplasty in which the deep stroma was left attached to the Descemet membrane. Highly reflective particles (arrows), which indicate the interface, are visible. Scale bar measures approximately 100 µm.

Activated keratocytes were found to occupy the deepest stroma immediately adjacent to the Descemet membrane. Activated keratocytes or repair fibrocytes are involved in the production of the repair extracellular matrix.\textsuperscript{18,19} Activated keratocytes were no longer visible in any corneas by 4 to 6 weeks after surgery in the deep group and 8 to 10 weeks after surgery in the near group.

In the first phase of wound repair and 4 days postoperatively, keratocytes disappeared in the area adjacent to the wound edge in both the deep and near groups. In the second phase of wound repair and 7 days after wounding, keratocytes surrounding the area of cell loss began to repopulate the wound area. It is unclear whether keratocytes surrounding the area of cell loss divide to replenish their numbers or whether new cells migrate to the wound edge. Whatever their source, these replacement cells would have been activated to become repair fibroblasts or myofibroblasts. The mechanism by which keratocytes initially disappear is not yet fully understood, though the fact that keratocytes disappear very quickly may indicate a mechanism of self-destruction, ie, apoptosis (programmed cell death).\textsuperscript{20}
face reflectivity and keratocyte activity. Our confocal microscopy also demonstrated evidence of new extracellular matrix deposition.

Studies of corneal wound healing have been conducted extensively in animal models and humans, especially in correlation with corneal transplantation, cataract extraction, radial keratotomy, laser-assisted in situ keratomileusis (LASIK), and photorefractive keratectomy.21 Those studies all showed that the cornea heals through a series of well-defined stages:22,23,24 active wound healing (initial corneal injury; proliferation and migration of surviving corneal epithelial cells, stromal keratocytes, and endothelial cells; and cellular differentiation with active removal, synthesis, and deposition of extracellular material) and tissue remodeling. Corneal function (ie, transparency and wound strength) usually returns to maximal, but not always normal, levels, after completion of the remodeling stage.

We admit that one of the weaknesses of our study is the lack of histological correlation with the changes observed. However, we plan to perform additional quantified studies correlating the ultrastructural, biomechanical, and histologic changes. Because of the smooth interface between the Descemet membrane and deep stroma, confocal microscopy in the deep group showed decreased intensity and reflectivity of activated keratocytes. Moreover, in the deep group, the healing comes only from the stromal side, which has plenty of keratocytes. The Descemet membrane is a glassy smooth membrane devoid of cells and thus does not share in the healing process, which is the advantage of reaching the level of the Descemet membrane; it minimizes the healing process and hence the haze. Thus, half of the surface area in maximum-depth anterior lamellar keratoplasty (the deep group) contributes little to nothing to haze or backscattered light (1-sided healing) compared with the near group, which showed increased intensity of highly reflective activated keratocytes owing to stroma-to-stroma healing (double-sided healing). Even with the removal of tissue down to the Descemet membrane and very minimal healing at the smooth interface in maximum-depth anterior lamellar keratoplasty (the deep group), there was no postoperative corneal bulge seen in any of the eyes. We have shown in another study on rabbits that the use of sutures induced a stronger healing at the corneal LASIK flap edge, increasing its mechanical strength and possibly preventing the late ectasia seen in some eyes that have undergone LASIK.24 The use of sutures may induce a foreign-body reaction at the flap edge, stimulating an influx of inflammatory cells, a transformation of myofibroblasts, and a synthesis of new ground stromal substance.25 With this finding, we have answered a very important question: Why do LASIK flaps become ectatic but deep lamellar transplants do not, even when the lamellar bed is dissected to the Descemet membrane? We postulated that the difference in outcomes between LASIK and lamellar keratoplasty was due to the strength contributed by the fibrotic scarring of sutures.

In LASIK, the lamellar interface usually shows minimal interface opacification. Also, in automated lamellar keratoplasty, haze formation is most often minimal at the smooth interface between the donor and recipient tissues. These observations suggest that the presence of a lamellar interface by itself may not cause interface opacification, but that the smoothness of the interface is critical. Dissection of deep stroma down to the Descemet membrane in maximum-depth anterior lamellar keratoplasty approaches the smoothness of a microkeratome dissection.25

In conclusion, in vivo confocal microscopy has shown the morphologic characteristics of the donor-recipient interface as well as keratocyte morphology and density after both near–Descemet membrane and maximum-depth anterior lamellar keratoplasty. The results of this study represent a step forward in understanding the healing of lamellar keratoplasties. We have shown that careful dissection and baring of the Descemet membrane in maximum-depth anterior lamellar keratoplasty reduce healing and subsequent haze, resulting in optimal corneal clarity, which would permit better quality of vision compared with other, conventional lamellar corneal operations.

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Figure 5. Confocal micrograph of an eye after deep anterior lamellar keratoplasty with the Descemet membrane completely bare. A, The wound margin (arrow) shows no keratocytes 4 days postoperatively. B, One week postoperatively, the cells with swollen stroma and oval, highly reflective keratocyte nuclei (arrows) are assumed to be activated keratocytes populating the wound margin. Scale bars measure approximately 100 µm.
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REFERENCES