Keratocyte Activity in Wound Healing After Epikeratophakia in Rabbits

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Epikeratophakia is a refractive surgical procedure for the correction of aphakia, high myopia, or keratoconus. To solve clinical problems associated with epikeratophakia, a basic knowledge of its postoperative healing process is needed. The authors investigated keratocyte activities, particularly cell proliferation and collagen synthesis, during wound healing after epikeratophakia in rabbits. Epikeratophakia was done on rabbit corneas with a homologous cryolathed keratolens. Ten, 16, 28, 45, 63, 90, 254, and 360 days after the operation, the corneas were excised, labeled with either \( { }^{3} \)H-thymidine (10 \( \mu \)Ci/ml) or \( { }^{3} \)H-proline (10 \( \mu \)Ci/ml) for 4 hr and examined histologically and by autoradiography. Keratocytes in keratolenses were killed during the freezing process. On postoperative day 10, a few keratocytes migrated to the edge of the keratolens from the host stroma. On days 16 and 28, keratocytes in the keratolens and host stroma near the junction between the host and the keratolens incorporated \( { }^{3} \)H-thymidine, suggesting active proliferation. The proliferating activity was no longer seen after day 45. The repopulation of keratocytes was almost complete on day 90 and gradually returned to normal through day 360. Keratocytes in the keratolens and host stroma beneath the keratolens showed a higher \( { }^{3} \)H-proline incorporation than the control from days 16-254 with the highest activity at around 4-9 weeks after surgery. These results suggest that remodeling of collagen fibers continues for a long postoperative period after epikeratophakia. Invest Ophthalmol Vis Sci 32:1837–1845, 1991

Epikeratophakia, a refractive surgical procedure, was first described by Kaufman in 1980. Since it is a relatively easy, safe, and effective operation, it has been used extensively to correct aphakia in infants, adult aphakic cases with difficulties of secondary implantation of an intraocular lens, or keratoconus. Although the usefulness of this procedure has been established, complications occasionally occur associated with delayed recovery or the fluctuation of postoperative visual acuity, delayed reepithelialization, or the opacification of the keratolens. To solve these problems, a basic knowledge of the postoperative healing process is needed. However, few morphologic studies have been done of the wound healing process after epikeratophakia. We investigated healing processes after epikeratophakia in rabbits, focusing on keratocyte activities, particularly cell proliferation and collagen synthesis.

Materials and Methods

The animals were maintained and treated in full compliance with the ARVO Resolution on the Use of Animals in Research. Albino rabbits (Japanese white) of both sexes weighing 2–3 kg were used for the experiment. Epikeratophakia was done with a cryolathed homologous graft (keratolens) in one eye of each rabbit; the untreated contralateral eye served as a control. The procedure of cryolathing keratolenses we used was not the traditional Barraquer method that uses a Steinway cryolathe. We froze rabbit corneas in liquid nitrogen and lathed frozen corneas to a lenticular shape 7.5 mm in diameter and 350-\( \mu \)m thick with a 6.0-mm optical zone and +10.0-diopter power. The endothelial–Descemet’s portion of the stroma was removed during cryolathing. We froze rabbit corneas in liquid nitrogen and lathed frozen corneas to a lenticular shape 7.5 mm in diameter and 350-\( \mu \)m thick with a 6.0-mm optical zone and +10.0-diopter power. The endothelial–Descemet’s portion of the stroma was removed during cryolathing. After this, the keratolenses were stored at -70°C and used for the surgery usually within 5 days. Rabbits were anesthetized by an intramuscular injection of ketamine hydrochloride (30 mg/kg), and drops of 0.4% oxybuprocaine hydrochloride were instilled into the eyes. After removal of the
epithelium with 100% ethanol and spartel, an incision of 0.2 mm in depth was made with a Barron double-bladed trephine (diameter, 7.0 mm), and circular wedge resection (less than 1-mm wide) was done along the inside of the incision. The keratolens was thawed in balanced salt solution and placed on the recipient's cornea with its edge tucked into the groove and sutured with four interrupted sutures and a continuous 10-0 nylon suture. The interrupted sutures were left in place, not removed. Erythromycin and prednisolone ointments were instilled into the eye until postoperative day 3. Of ten eyes of ten rabbits that underwent the surgery, two eyes of two rabbits resulted in keratolens opacification, possibly due to delayed reepithelialization. Eight eyes of eight rabbits in which keratolens remained clear were enucleated on days 10, 16, 28, 45, 63, 90, 254, and 360 after surgery after killing the rabbits with a lethal dose of sodium pentobarbital. The corneas were removed from the enucleated eyes and labeled with either 3H-thymidine (10 μCi/ml) or 3H-proline (10 μCi/ml) (Amersham, Buckinghamshire, England) in Dulbecco's minimum essential medium (1 ml/cornea; Gibco, Grand Island, NY) at 37°C for 4 hr. The 3H-labeled corneas were examined histologically and by autoradiography. Autoradiography was done as described previously.14 The corneas were fixed, and paraffin sections (6-μm thick) were prepared. The sections were coated with nuclear track emulsion Konica NR-M2 (Konica, Tokyo, Japan), exposed for 3 weeks, developed with Fuji Lendole (Fuji Photo Film Co., Tokyo, Japan), and fixed with Fuji Lenfix. The sections were then stained with hematoxylin and eosin. They were examined histologically under a light microscope.

To examine the effect of 100% ethanol on host corneas, epithelium was removed from a normal rabbit cornea with 100% ethanol. Erythromycin and prednisolone ointments were instilled into the eye until the third day. The cornea was examined histologically on the 7th day.

Results

Autoradiography of Corneas Labeled With 3H-Thymidine

Keratocytes in the keratolens were killed during the process of freezing (Fig. 1). On the 10th postoperative day, enlarged, activated keratocytes started to migrate into the periphery of the keratolens from the host stroma. Some of them incorporated 3H-thymidine, suggesting proliferation (Fig. 2). The central area of the keratolens lacked keratocytes. Keratocytes were sparse in the anterior area of the host stroma immediately under the keratolens. Adjacent keratocytes were
activated and some cells incorporated $^3$H-thymidine (Figs. 3, 4). On the 16th day, more keratocytes had migrated into the keratolens and incorporated $^3$H-thymidine, suggesting active proliferation (Fig. 5). On the 28th day, keratocytes had migrated into the center of the keratolens. They incorporated $^3$H-thymidine, showing that proliferation of keratocytes was still present at this stage (Fig. 6). The population of keratocytes was still sparse in the anterior area of the keratolens beneath the epithelium (Fig. 6). From day 45–360 postoperatively, keratocytes containing silver grains were no longer seen in either the keratolens or host stroma. The population of keratocytes was still sparse in the keratolens and host stroma on day 45 (Fig. 7). Repopulation of keratocytes in the keratolens was not complete even by the 63rd day (Fig. 8), but it was almost complete after day 90 in the keratolens and anterior area of the host stroma beneath the kerato-

Fig. 3. $[^3]$HThymidine autoradiogram of rabbit cornea on the 10th day postepikeratophakia (original magnification ×50). Keratocytes in the keratolens (k) and in the host stroma (h) are incorporating $[^3]$Hthymidine (arrows). The interface between the keratolens and the host stroma is clearly observed (arrowheads), and an acellular area (***) is present in the host stroma beneath the interface.

Fig. 4. $[^3]$HThymidine autoradiogram of rabbit cornea on the 10th day postepikeratophakia (original magnification ×200). Higher magnification of the host stroma of Figure 4. Keratocytes are enlarged and round-shaped. Some of them are incorporating $[^3]$Hthymidine (arrowheads).

Fig. 5. $[^3]$HThymidine autoradiogram of rabbit cornea on the 16th day postepikeratophakia (original magnification ×50). There are many keratocytes in the intermediate area of the keratolens (k), suggesting that the migration of keratocytes starts from that area. Keratocytes are accumulating along the gap (****) between the keratolens (k) and the host tissue (h).
lens (Figs. 9, 10). There was no evidence of keratocyte migration perpendicular to the interface between the keratolens and the host stroma.

**Autoradiography of Corneas Labeled With \( ^{3}H \)-Proline**

The \( ^{3}H \)-proline uptake by keratocytes in the host stroma beneath the keratolens started to increase on day 16, reached a peak on day 28, and then declined. However, even on day 254, the uptake was still higher than in the control (normal cornea). On day 360, it returned to the control level (Fig. 11). Keratocytes in the keratolens were activated and incorporated \( ^{3}H \)-proline as actively as those in the host stroma on day 28 (Fig. 12). The \( ^{3}H \)-proline uptake by keratocytes in the keratolens and that in the host stroma was almost the same as in other stages. On day 63, keratocytes in the central area of the host stroma beneath the keratolens actively incorporated \( ^{3}H \)-proline; those in the peripheral area of the host stroma did not (Fig. 13). Sections of the cornea on the 45th postoperative day revealed keratocytes accumulating along the interface between the keratolens and the host stroma (Fig. 14).

**Histology of Normal Cornea on the 7th Day After Removal of Epithelium with Ethanol**

To examine the effect of 100% ethanol on corneas, epithelium was removed from the normal cornea with 100% ethanol, and after 7 days, the cornea was examined histologically. The results demonstrated thinly regenerated epithelium and irregularly organized collagen fibers and keratocytes in the stroma.
undamaged. Only a few studies have reported the time course of healing after epikeratophakia. One study found that in monkey eyes repopulation of keratocytes in the keratolens started on the 9th day after operation and was completed after 6 weeks; the keratocytes were morphologically normal. Rich et al did keratophakia and inserted a cryolathed keratolens into the cornea interlamellarily; they first found keratocytes in the keratolens on day 10 post-operatively. Others examined monkey eyes histologically 22 and 25 months after epikeratophakia and suggested that keratocytes might migrate over the host Bowman’s membrane through the junction between the host stroma and the keratolens to repopulate the keratolens gradually from the deeper area. Another study reported that in baboon eyes 5 months after epikeratophakia, activated keratocytes were observed along the interface between the host stroma and the keratolens. However, these studies were all based on static, morphologic observation and speculation. We inves-

Discussion

To provide accurate knowledge to help with the clinical problems associated with epikeratophakia, we investigated wound healing after experimental epikeratophakia in rabbits. Since epikeratophakia is a surgical method of refractive correction, a keratolens is prepared by freezing and lathing the donor cornea to an appropriate shape and refractive power. It has been reported that during the process of freezing, donor keratocytes are killed (confirmed in our study) and that the diameter and interfibrillar spaces among collagen fibers become larger than normal with changes in Bowman’s membrane. Therefore, wound healing after epikeratophakia is very different from that after penetrating keratoplasty in which donor epithelium and stromal keratocytes and collagen fibers are

Fig. 8. \[^{3}H\]Thymidine autoradiogram of rabbit cornea on the 63rd day postepikeratophakia (original magnification X50). Keratocytes return to spindle-shaped and repopulate relatively well. A narrow acellular area (*) is still present under the epithelium. Radioactive keratocytes are not seen.

However, there was no area in the stroma where the population of keratocytes was sparse (Fig. 15).

Fig. 9. \[^{3}H\]Thymidine autoradiogram of rabbit cornea on the 90th day postepikeratophakia (original magnification X50). The repopulation of keratocytes in the keratolens is almost complete. There is no radioactive keratocyte.
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Fig. 10. [3H]Thymidine autoradiogram of rabbit corneas on days 254 and 360 postepikeratophakia (original magnification X50). There population of keratocytes in the keratolens gradually returned to normal.

Treated keratocyte activities, particularly proliferating activity and collagen synthesis during wound healing after epikeratophakia in rabbits, by labeling corneas with [3H]-thymidine or [3H]-proline. Since the rabbit eye is unlike the human eye in that it does not have a Bowman's layer and the preparation of keratolenses used in this experiment was different from that for human patients, the wound healing process shown in this study may differ from that in the human eye. Therefore, the time course of the events shown here cannot be extrapolated directly to humans. However, our results may provide useful information and a better understanding of the healing process after epikeratophakia.

Our results are summarized and illustrated in Figure 16. Keratocytes started to migrate into the keratolens from the host stroma on day 10 after surgery through the host-keratolens junction where the edge of the keratolens has been tucked into the groove of the host stroma. Mitotic activity of keratocytes, not seen in normal corneas, was observed after this stage. Some keratocytes that had migrated into the keratolens and those in the host stroma near the host-keratolens junction incorporated [H]-thymidine. After 16-28 days, more keratocytes migrated into the center of the keratolens and proliferated actively. After the

Fig. 11. [3H]Proline autoradiograms of rabbit corneas (the central area of the host stroma) on days 16 to 360 postepikeratophakia (X200). In the normal cornea (C), keratocytes incorporate almost no [3H]-proline. [3H]-Proline uptake by keratocytes in the host stroma starts to increase on day 16, reaches a peak on day 28, and then declines. However, the uptake is still higher than in the normal cornea (c) even on day 254. Keratocytes are most enlarged and round-shaped on day 28.
Fig. 12. $[^{3}H]$Proline autoradiogram of rabbit cornea (the central area of the keratolens) on the 28th day postepikeratophakia (original magnification $\times 200$). Keratocytes in the keratolens incorporate $[^{3}H]$proline as actively as those in the host stroma.

45th day, the mitotic activity of keratocytes was not observed, suggesting that the peak proliferative activity of keratocytes was around 2–4 weeks after surgery. The migration of keratocytes started from the intermediate area of the keratolens. They did not infiltrate from the deep area of the keratolens. However, at around 2 weeks postoperatively, keratocytes accumulated along the interface between the keratolens and the host tissue, and the population of keratocytes was sparse under the epithelium. This acellular area under the epithelium was still observed in the cornea on day 63 after surgery, but it was almost completely repopulated with keratocytes by day 90. An acellular area

Fig. 13. $[^{3}H]$Proline autoradiograms of rabbit cornea on day 63 postepikeratophakia (original magnification $\times 200$). Keratocytes in the central area of the host stroma (A) where the keratolens covers incorporate $[^{3}H]$proline actively, whereas those in the peripheral area of the host stroma (B) do not.

Fig. 14. Light micrograph of rabbit cornea on day 45 postepikeratophakia (original magnification $\times 50$). Keratocytes accumulate along the interface between the keratolens and the host stroma (arrows).

Fig. 15. Light micrograph of rabbit cornea 7 days after the removal of the epithelium with 100% ethanol (original magnification $\times 50$). Regenerated epithelium is thinner than normal. Keratocytes and collagen fibers under the epithelium are disorganized, but there is no acellular area beneath the epithelium.
Fig. 16. Illustrations demonstrating wound healing after epikeratophakia. Keratocytes in the host stroma migrate into the keratolens through the host–keratolens junction. Proliferating activity of keratocytes is observed from day 16 to day 28 in the keratolens and the host stroma. Collagen synthesis by keratocytes is preceded by their migration and reaches a peak on around day 28, then declined. But it continues even on day 254 postoperation. Acellular areas are observed under the epithelium and under the host–keratolens interface from the early postoperative stage. The repopulation of keratocytes in the keratolens is almost complete after day 90.

was also seen in the anterior part of the host stroma immediately beneath the host–keratolens interface from the early postoperative stage onward. This acellular area was repopulated with keratocytes after 63 days.

The acellular area under the epithelium might affect the process of epithelial regeneration. Since keratocytes were killed during the process of freezing the keratolens, reepithelialization of the keratolens (composed only of damaged collagen fibers) may differ from that of the usual epithelial defect and may take longer. Therefore, activation of keratocytes might be inhibited, resulting in an acellular area beneath the epithelium. The acellular area in the host stroma under the keratolens can be explained partly by the effect of ethanol. To examine the effect of ethanol, epithelium was removed from normal rabbit corneas with 100% ethanol, and after 7 days the corneas were examined histologically. Although regenerated epithelium was thinner than normal and keratocyte and collagen fibers were disorganized, there was no acellular area under the epithelium. This suggests that ethanol damages keratocytes, but acellular areas are not caused solely by it. Damaged host keratocytes might be killed easily by hypoxia (caused by being covered with the keratolens). This may account for the existence of the acellular area under the keratolens.

Therefore, for faster repopulation of keratocytes in the keratolens, it is important to minimize the procedure of removing the host epithelium with ethanol, to have an appropriate width of the host–keratolens junction, and to promote epithelial regeneration of the keratolens.

Our results showed that, during wound healing after epikeratophakia, collagen synthesis in keratocytes that had repopulated the keratolens was the same as that in keratocytes in the host stroma under the keratolens. It started to increase on day 16 postoperatively when the repopulation of keratocytes also increased, with its peak at around 4–9 weeks after surgery; then it declined. However, it was still more active than in the control eye on day 90 and even on day 254 postoperatively, suggesting active remodeling of the collagen fibers. This may in part be the cause of postoperative complications of delayed recovery or fluctuation of visual acuity. Keratocytes tended to accumulate along the interface between the host and the keratolens on day 16. There was marked accumulation on day 45, suggesting that keratocytes participate actively in the adhesion and healing of the host tissue and the keratolens. Collagen synthesis was preceded by the proliferation and repopulation of keratocytes. These results suggest that keratocytes were engaged in remodeling collagen fibers in the keratolens, healing damaged collagen fibers in the host stroma, and creating adhesions in the host tissue and the keratolens.

Key words: epikeratophakia, corneal wound healing, keratocyte proliferation, collagen synthesis, autoradiography

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References


