Corneal Epithelial Wound Healing in the Absence of Limbal Epithelium

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Corneal epithelial stem cells are thought to be at the limbus. The limbal epithelium was surgically removed in 12 New Zealand white rabbits. After 6 months, four showed mild vascularization. To challenge the remaining proliferative reserve, two consecutive 7.5-mm epithelial woundings were created 3 weeks apart in 11 limbal-deficient corneas and 11 controls. After the first wounding, five of the limbal-deficient corneas showed delayed healing, and seven became moderately vascularized; the controls healed normally. After the second wounding, eight experimental corneas showed intense vascularization; the controls did not. Recurrent erosions with delays in healing were noted in nine experimental animals but not in controls. Flat-mount preparation and impression cytology revealed centripetal migration of conjunctival epithelium with goblet cells onto the experimental corneas. These results indicate that only limited proliferative capacity of corneal epithelium remains in the absence of limbus. The constellation of delayed healing with recurrent erosion, corneal vascularization, and conjunctival epithelial ingrowth can be considered possible signs of limbal stem cell dysfunction.


In postnatal development, cells in any defined tissue that has active cellular renewal and differentiation can be classified into either of the two cellular compartments, proliferative or nonproliferative, depending on the presence or absence of mitotic activity. Numerous cell-kinetic studies of hematopoietic systems and such epithelial tissues as intestinal epithelium, epidermis, and seminiferous epithelium indicate that the proliferative compartment includes stem cells and transient amplifying cells. Stem cells are slow cycling and quiescent with a low mitotic activity in the normal steady state. However, they are long lived and can be activated by tissue demand for cellular regeneration. After a round of mitosis, stem cells give rise to transient amplifying cells, which are rapid cycling and can amplify cell mass effectively by limited rounds of mitosis. At a critical point, transient amplifying cells will cease mitosis and enter a nonproliferative compartment, committed to terminal cellular differentiation. Therefore, in all self-renewing tissues, stem cells can be regarded as the origin of cell lineage and the ultimate source of cellular proliferation and differentiation.

It is well known that corneal epithelium is well differentiated and has a rapid self-renewing capacity. Previous studies thought that the proliferative source of this tissue resided at the basal cell layer or in the corneal periphery, based on the observation of centripetal cellular movement. The exact location of the stem cells of corneal epithelium was unclear until Schermer et al. showed the absence of a differentiation marker, a 64-kD corneal epithelial keratin, in the limbal basal cells, which suggested that limbal basal epithelium may contain the corneal stem cell. Additional evidence was provided by Ebato et al. who showed that limbal epithelial cells grow better than peripheral or central corneal epithelia in explant cultures and by Cotsarelis et al. who showed that limbal basal cells can be selectively stimulated by the topical application of a tumor promoter and by wounding the central corneal epithelium.

Extrapolating from these data, we hypothesized that while the stem cells are in the limbus, the corneal basal epithelium contains the transient amplifying cells and that all the suprabasal cells of the cornea are postmitotic and committed to terminal differentiation. To examine this concept, we chose to compare the wound healing of central corneal epithelial defects when the limbal epithelium was either intact or removed.
Materials and Methods

All animal experiments conducted in this study conformed to the ARVO Resolution on the Use of Animals in Research.

Surgical Limbal Removal in Rabbits

To ensure complete removal of the limbal epithelium, a ring lamellar dissection including 2 mm of peripheral cornea and 3 mm of perlimbal conjunctiva was done on one eye of 12 New Zealand albino rabbits. After marking with a trephine, a No. 69 Beaver blade (Boston, MA) was used to create a partial-thickness, circular corneal incision at the peripheral cornea, 2 mm within the limbus. Lamellar dissection toward the limbus was then done with a No. 66 Beaver blade. A 360° conjunctival peritomy was made 3 mm beyond the corneolimbal junction and dissected toward the limbus. The entire limbal zone was then excised with a pair of scissors in a ring. Topical gentamicin sulfate ointment was applied daily for 3 days. All rabbits were examined weekly and photographed monthly for evidence of corneal vascularization or recurrent epithelial erosion over 6 months. At the end of 6 months, one representative rabbit was killed, and the corneoscleral button was removed for flat-mount preparation.

Central Corneal Epithelial Wounding

To investigate corneal epithelial wound healing in the absence of limbal epithelium, a central 7.5-mm corneal epithelial defect was created 6 months after limbal removal in the remaining 11 corneas of the experimental group and in another 11 control corneas with normal, intact limbus. After marking with a trephine into the superficial stroma, the central corneal epithelium was debrided with a surgical blade. The wounds were examined daily after 1% fluorescein staining. Three weeks after the first debridement, a second 7.5-mm central corneal wound was made in the same manner in both groups. Wound healing was again examined daily with fluorescein staining. Photographs were taken daily for the first 3 days, on day 7 of the first wounding, and on day 10 of the second wounding. Recurrent epithelial erosions were recorded if the previously healed area became enlarged or eroded based on serial fluorescein stainings.

Studies of Wound Healing

To examine the occurrence of corneal vascularization, external photographs taken before and after each of the two consecutive debridements were compared in both the experimental and control groups. To measure the healing rate, serial fluorescein external photographs of both groups were projected onto a screen at a fixed distance. The area of the subepithelial trephine mark and the area of epithelial defect outlined by the fluorescein staining were measured with a Zeiss Videoplan 2 image analyzer (Kontron, Eching, Germany). Using the 7.5-mm trephine-marked area as an internal standard, the area of epithelial defect was derived for each cornea. The data of each group were pooled and analyzed by paired student t-test and by Wilcoxon rank-sum test, with the assistance of William Feuer.

To detect the emergence of conjunctival epithelial ingrowth on the corneas, corneal flat-mount preparations17 and impression cytologies18 were done as previously described. Flat-mount preparations were stained with periodic acid-Schiff (PAS) to detect the presence of conjunctival goblet cells on the corneas. Impression cytology was stained with a combination of PAS and Papanicolaou staining to study the epithelial phenotype.

Results

After surgical removal of the limbal tissue, including 2 mm of the peripheral cornea and 3 mm of the perlimbal conjunctiva, the wounds of all 12 rabbit eyes healed in 3–5 days without any complications. To determine if the remaining central corneal epithelium would have any problem in maintaining the ocular surface integrity, these rabbits were followed for 6 months. During this time, no evidence of epithelial breakdowns such as erosions or epithelial defects was noted. At the end of 6 months, four of 12 (33%) corneas showed vascularization, one with dense pannus formation and three with mild peripheral vascularization.

A flat mount of the corneoscleral button of one of the three rabbits with mild vascularization was prepared at the end of the 6-month follow-up period. The result revealed the emergence of goblet cells at the peripheral cornea (Fig. 1A). In contrast, no goblet cells were found on the cornea of a normal control, which did not have surgical removal of the limbus (data not shown).

To challenge the proliferative capacity of the residual corneal epithelium, two consecutive 7.5-mm central corneal epithelial defects were created in both the experimental and control groups (n = 11, each). After the first debridement, the pooled data of the residual-defect areas of the corneas with limbal removal was significantly larger than those of the control corneas with intact limbus (Fig. 2). This difference could be detected from days 2–7 after debridement (P < 0.05, Fig. 2).
Fig. 1. Flat preparation of the corneal button 6 months after limbal removal (a) showing migration of the goblet cells onto the peripheral cornea. Progressive migration of goblet cells onto the central cornea was noted 3 weeks after the second wounding (b). Arrows indicate the corneolimbal junction.

Regression of the kinetic curves shown in Figure 2 was 0.43 mm²/hr and 0.50 mm²/hr for the experimental and control corneas, respectively. As demonstrated in the serial photographs of one representative case of each group (Fig. 3), the normal control cornea healed uneventfully without any erosion in 7 days, but the experimental cornea with limbal removal showed delayed healing and corneal epithelial erosion. Recurrent epithelial erosion was noted in five of 11 (45%) experimental corneas but in none of the controls (P < 0.1).

Three weeks after the first wounding, a second 7.5-mm central epithelial debridement was done. The wound-healing rate was also noted to be significantly slower in the corneas with limbal removal compared with the control group from postoperative days 2–10 (P < 0.05, Fig. 4). The kinetic healing curve after the second wounding was biphasic, which differed from the linear healing pattern of the first wounding (Fig. 4). In the second wounding, the healing rate was rapid during the first 3 days in both the experimental and control groups but slowed thereafter in the experimental group. The healing rate of the first phase of the second wounding healed faster than that of the first wounding in both the experimental and control groups (Fig. 2 compared with Fig. 4). When the linear portion of the second wound healing curve (from days 0–3) was subjected to linear regression, the healing rate was calculated to be 0.60 mm²/hr and 0.79 mm²/hr for the experimental and control corneas, respectively (Fig. 4). The delayed healing noted in the second phase after day 3 in the experimental group could be attributed to the recurrent epithelial breakdowns that were frequently observed in the experi-
FIRST CENTRAL CORNEAL WOUNDING

Fig. 2. Areas of remaining epithelial defect during 1 week of healing after the first 7.5 mm diameter circular wound of corneal epithelium in the experimental group with limbal removal (●) and the normal control (○). The extension bar represents the standard error. P values were derived from the analysis by Wilcoxon Rank Sum Test. A significant delay of wound healing was noted in the experimental group from day 3 to day 7.

mental corneas with limbal removal (Fig. 5). Delayed healing due to recurrent epithelial erosion was noted in ten (90%) of 11 experimental corneas compared with none in the controls (P < 0.01).

As stated, peripheral vascularization was observed in four corneas (33%) 6 months after limbal removal (Figs. 6D,G,J). However, 1 week after the first central corneal epithelial wounding, the extent of vascularization progressed toward the central cornea (Figs. 6E,H,K) and advanced further 1 week after the second corneal debridement (Figs. 6F,I,L). Progression of corneal vascularization was noted in seven (64%) of 11 corneas with limbal removal after the first wounding and increased to eight (73%) after the second wounding. No vascularization was noted in the control corneas after either the first or second woundings (Figs. 6A–C). When the incidence of corneal vascularization in the experimental group was compared with that of the control, it was statistically significant for the first and second woundings (P < 0.05, respectively).

To study if there was any progression of conjunctival epithelial ingrowth, both corneal flat-mount preparations and impression cytology were done. The flat-mount preparations of the experimental corneas obtained 2 weeks after the second corneal wounding showed that goblet cells had migrated onto the central cornea with an increasing density (Fig. 1B). Impression cytology taken from the limbal region confirmed that after the second consecutive woundings, no goblet cells migrated to the corneal surface of the normal controls (Figs. 7A–B). However, goblet cells were found in a high number after the first (Fig. 7C) and the second wounding in corneas with limbal removal (Fig. 7E). Additionally, both goblet and nongoblet conjunctival epithelial cells were found on the central corneal area after the second wounding in these corneas (Figs. 7D,F).

Discussion

The limbal location of corneal epithelial stem cells is a new concept. It implies that limbal basal epithelium serves as the ultimate source of cellular proliferation and differentiation by providing an unlimited supply of transient amplifying cells. Through a high, although limited, proliferative capacity of the transient amplifying cells, the demand for rapid self-renewal of the corneal epithelium can be achieved. Because of such a unique feature, the perilimbal zone also precludes the invasion of conjunctival epithelium onto the corneal surface under normal circumstances. A similar concept of growth pressure was first mentioned by Friedenwald.19 When the limbal zone was surgically removed as shown in our study, the features of abnormal wound healing ensued and encompassed delayed healing with recurrent epithelial erosion, corneal vascularization, and conjunctival epithelial ingrowth. These results also substantiate the belief that the limbal epithelium is the location of the stem cells for corneal epithelial proliferation and differentiation, a concept first proposed by Schermer et al.14

As we demonstrated, initial removal of the limbal stem cells does not lead to a notable difficulty in corneal epithelial wound healing. The remaining transient amplifying cells, presumably the corneal basal cells, were evidently still able to maintain the central corneal epithelial cell mass and integrity for at least 6 months. To explain the duration of time required for the corneal epithelium to show possible decompensation under such a circumstance, we refer to several previous investigations.10–13 Centripetal cellular movement by the peripheral corneal epithelium is responsible for the healing of traumatic loss of central corneal epithelium, such as an epithelial defect.7–9 However, even in a less traumatic situation, eg, penetrating keratoplasty or lamellar keratoplasty in which the central corneal epithelium is substituted by the donor tissue, the centripetal movement of the surrounding host epithelium to replace the donor derivatives is well documented by the elegant work of Kinoshita et al.11 They observed a gradual dilution of sex chromatin on the female donor graft by the male recipient over 12 weeks. When their data are extrapolated to the end point of complete replacement, it is
Fig. 3. Comparison of external photographs of corneal epithelial wound healing with fluorescein staining after the first 7.5 mm central corneal wounding between one representative case of the control (left column) and of the experimental group (right column). Delayed healing was noted in the experimental group.
The clinical observations of centripetal migration of the epithelial dots in penetrating keratoplasty 10 and a diameter of 10-12 mm is estimated at about 1 yr. These data are also consistent with the relatively low incidence of epithelial rejection compared with other types of graft rejection 1 yr after penetrating keratoplasty.12 Even under normal atraumatic desquamation, Buck13 also detected centripetal migration of superficial corneal epithelial cells in mice and measured a rate of 17 μm per day. Variations among different species may exist. Nonetheless, when this migration rate (17 μm/day) is applied to the rabbit11 and human10,12 conditions, the total replacement of a corneal epithelium with a diameter of 10-12 mm is estimated at about 1 yr. Therefore, in the present experiment, we could expect that the epithelial breakdown might begin in 1 yr when the remaining proliferative reserves of the transient amplifying cells are exhausted and no such new cells have been generated by the limbal stem cells. The fact that the corneal flat mount prepared 6 months after limbal removal showed the emergence of conjunctival goblet cells at the peripheral cornea (Fig. 1A) further suggests a deficiency of limbal stem cells that might later lead to inadequate corneal epithelial regeneration. To substantiate that limbal basal epithelium, instead of peripheral corneal epithelium, contains the stem cell population and to distinguish the functional role between stem cells and transient amplifying cells in wound healing, we challenged the remaining cellu-

![Fig. 4. Areas of remaining epithelial defect after the second 7.5 mm diameter circular wounding of corneal epithelium in the experimental group with limbal removal (●) and the normal control (○). The extension bar represents the standard error. P values were derived from the analysis by Wilcoxon Rank Sum Test. A significant delay of wound healing was noted in the experimental group from day 3 to day 7.](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933385/) interesting to note that the entire process might have taken about 1 yr. These data are also consistent with the clinical observations of centripetal migration of the epithelial dots in penetrating keratoplasty10 and with the relatively low incidence of epithelial rejection compared with other types of graft rejection 1 yr after penetrating keratoplasty.12 Even under normal atraumatic desquamation, Buck13 also detected centripetal migration of superficial corneal epithelial cells in mice and measured a rate of 17 μm per day. Variations among different species may exist. Nonetheless, when this migration rate (17 μm/day) is applied to the rabbit11 and human10,12 conditions, the total replacement of a corneal epithelium with a diameter of 10-12 mm is estimated at about 1 yr. Therefore, in the present experiment, we could expect that the epithelial breakdown might begin in 1 yr when the remaining proliferative reserves of the transient amplifying cells are exhausted and no such new cells have been generated by the limbal stem cells. The fact that the corneal flat mount prepared 6 months after limbal removal showed the emergence of conjunctival goblet cells at the peripheral cornea (Fig. 1A) further suggests a deficiency of limbal stem cells that might later lead to inadequate corneal epithelial regeneration. To substantiate that limbal basal epithelium, instead of peripheral corneal epithelium, contains the stem cell population and to distinguish the functional role between stem cells and transient amplifying cells in wound healing, we challenged the remaining cellu-

lar reserve by creating two consecutive 7.5-mm central epithelial debridements. With respect to the proliferative reserve, there was a statistically significant delay of wound healing and recurrent erosion in the experimental group compared with the normal controls (Figs. 2,4). This result indicates that the residual transient amplifying cells in the peripheral cornea were not sufficient to maintain the entire corneal epithelial cell mass. Since transient amplifying cells are known to have a high mitotic activity and a short cell cycle, their insufficiency to attain the proliferative goal can be explained by their shorter limited life span. We also observed that there was a statistically significant delay of wound healing and worsening of recurrent erosions when the second wounding was compared with the first. This suggests that there is further depletion of transient amplifying cells after the first wounding in the experimental group, which could not be replenished due to the removal of limbal stem cells. The fact that the normal controls could withstand two such consecutive epithelial debridements indicates that the proliferative source had not been violated.

It is also interesting that the initial healing rate of the second wounding was faster than that of the first in both groups. This finding indicates that the residual proliferative cellular compartment had been activated by the first wounding and the newly (younger) regenerated cells are more readily responsive to the demand for cellular proliferation when the second wounding was induced. Similar findings have also been observed by Srinivasan et al.20 when they measured the healing rate in rabbit corneas receiving repetitive corneal epithelial trauma. The fact that this initial rapid healing could only last for 3 days in the experimental group and was immediately followed by markedly delayed healing further supports the notion that the residual proliferative cells were indeed transient amplifying cells and not stem cells.

With respect to the differentiative reserve, we also observed that limbal removal led to a significant increase of conjunctival epithelial ingrowth, which was characterized by the invasion of conjunctival goblet cells onto the central cornea (Figs. 1,7). The progressive centripetal ingrowth of conjunctival epithelium with goblet cells signifies the deranged junctional epithelial barriers between corneal and conjunctival epithelia. Under normal circumstances, such an epithelial barrier is presumably operated by the high mitotic activity of transient amplifying cells derived from limbal stem cells. Due to the limited life span of these cells, the growth pressure of the perilimbal zone will eventually rely on the limbal stem cells, which can continuously generate newer and more active proliferative transient amplifying cells. In the absence of
Fig. 5. Comparison of external photographs of corneal epithelial wound healing with fluorescein staining after the second 7.5 mm central corneal wounding, between one representative case of the control (left column) and of the experimental group (right column). Delayed healing with recurrent epithelial breakdown was frequently noted in the experimental group. The latter can be illustrated by enlarged area of fluorescein staining in 10 d as compared to 3 d.
Fig. 6. External photographs of one control normal cornea (upper panel), and three representative experimental corneas with limbal removal (bottom three panels). No vascularization was noted in the control cornea 1 week following the first (b) and second (c) wounding as compared to the preoperative state (a). Minimal vascularization indicated by arrows was noted in some experimental corneas (d, g, j) 6 months following the limbal removal, progression of the corneal vascularization towards the central cornea became apparent 1 week after the first wounding (middle panel, e, h, k) and 1 week after the second wounding (right panel, f, i, l).

limbal stem cells, the healing of the central corneal epithelial defect triggers the ingrowth of surrounding conjunctival epithelium.

In this study, we observed corneal vascularization 6 months after limbal removal in four of 12 (33%) rabbits. After the first and second wounding, the incidence of vascularization increased to 64% and 73%, respectively, as did the severity. This result indicates that the deficiency or dysfunction of limbal stem cells would also lead to increasing corneal vascularization. It has long been recognized that corneal epithelial defects in the limbus invariably heal without any vascularization. But if the defects extend beyond the limbus, variable incidences, ranging from 14–65%, of
Fig. 7. Impression cytology specimen from the peripheral corneal/limbal region (left column) and the central cornea (right column) of the normal control (a, b) and of the experimental corneas (c-f) following two woundings. The normal control did not show any goblet cell migration to either the peripheral (a) or central (b) cornea. In contrast, the centripetal migration of numerous goblet cells (GC), indicated by large open arrows, onto the peripheral and central corneas was noted in the experimental corneas following the first (c, d) and second (e, f) wounding. The solid arrows in (a), (c), and (e) indicate the anatomic function of cornea and limbus.

corneal vascularization and conjunctival epithelial ingrowth have been reported. Ref. 21-24 Recently, we noted that the total removal of limbal and corneal epithelia resulted in a high (96%) incidence of corneal vascularization in 54 rabbit eyes. Ref. 23 Furthermore, conjunctival transplantation including the limbal epithelium, a procedure called limbal transplantation, could restore the corneal epithelial phenotype in the
damaged corneal surface more effectively than conjunctival transplantation alone. After limbal transplantation, the previously vascularized corneas showed a progressive decrease of vascularization, a phenomenon also observed in human patients. Taken together, these observations suggest that on the corneal surface, conjunctival epithelial ingrowth is accompanied by corneal vascularization. This concept is also supported in our recent study of partial limbal deficiency. To explain this association, one can speculate that conjunctival epithelium may produce certain inflammatory or angiogenic mediator(s) that are essential to maintain its phenotypic expression.

In summary, we showed that corneal epithelial wound healing was impaired in the absence of limbal stem cells. Total removal of limbal stem cells can lead to (1) delayed wound healing with recurrent epithelial breakdowns, (2) corneal vascularization, and (3) conjunctival epithelial ingrowth (conjunctivalization). This constellation of abnormal features can thus be regarded as typical signs of limbal stem cell deficiency (dysfunction). Similar findings have been observed clinically in such ocular surface disorders as chemical injuries, Stevens-Johnson syndrome, aniridia, and some contact lens-induced keratopathy. We speculated that dysfunction of the limbal stem cells might be the basis for these disorders. Clinically, it is therefore important to recognize such a disease entity and its pathophysiology. Use of the unique property of limbal stem cells, eg, limbal transplantation, can enhance our ability to manage these disorders. Further exploration of the regulatory mechanism of the limbal stem cells will help our understanding of other ocular surface disorders that have various other manifestations of stem cell dysfunction.

**Key words:** cornea, epithelium, limbus, stem cells, wound healing

### References


