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Examination of the Restoration of Epithelial Barrier Function Following Superficial Keratectomy

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Abstract

The goal of the present study was to determine the rate of restoration of the corneal epithelial barrier following a superficial keratectomy using a functional assay of tight junction integrity. Adult Sprague-Dawley rats were anesthetized and a 3-mm superficial keratectomy was performed. The eyes were allowed to heal from 4 hours to 8 weeks and the rate of epithelial wound closure was determined. To examine the restoration of the barrier function, EZ-Link Sulfo-NHS-LC-Biotin (LC-Biotin) was applied to all eyes, experimental and control, for 15 minutes at the time of sacrifice. This compound does not penetrate through intact tight junctions. Indirect immunofluorescence was performed with anti-laminin, a marker of basement membrane; fluorescein-conjugated streptavidin to detect the biotinylated marker; and anti-occludin and anti-ZO-1, markers of tight junctions.

Epithelial wound closure was observed at 36–42 hours after wounding. LC-Biotin did not penetrate the intact epithelium. Upon wounding, LC-Biotin penetrated into the stroma subjacent and slightly peripheral to the wound area. This pattern was present from 4–48 hours post-wounding. The area of LC-Biotin localization decreased with time and the functional barrier was restored by 72 hours. Occludin and ZO-1 were present at all time points. The number of cell layers expressing these proteins appeared to increase at 48 and 72 hours. Continuous laminin localization was not observed until at least 7 days after wounding. Barrier function is restored within 1–1.5 days after epithelial wound closure. The loss of barrier function does not extend beyond the edge of the original wound. The restoration of barrier function does not appear to correlate with reassembly of the basement membrane in this model.

Keywords

Barrier Function; Cornea; Epithelium; Tear Film; Basement Membrane; Stroma; Biotin; Tight Junction

1. Introduction

The corneal epithelium is the first line of defense against the outside world. Normally, the intact epithelium with its associated tear film, inhibits macromolecules, such as, viruses and bacteria from entering the cornea.(Lee, et al., 2003; Lu, et al., 2001; Price-Schiavi, et al., 1998; Yi, et al., 2000) The epithelial barrier function is thought to be primarily the product of the intracellular junctions termed tight junctions or zonula occludins.(Sugrue and Zieske, 1997; Wang, et al., 2004; Yi, et al., 2000) These junctions consist of several intracellular proteins including occludin, ZO-1 and ZO-2(Anderson, 2001; Schneeberger and Lynch,

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2004) and are localized between the superficial cells. These junctions are resistant to penetration by molecules greater than 182 Da.(Huang, et al., 1989) More recently, it has also been proposed that the epithelium-associated mucins also make up a portion of the barrier function.(Gipson, 2004; Price-Schiavi, et al., 1998) Indeed, removal of these mucins results in a decrease in the barrier function.(Dursun, et al., 2000)

Normally, the corneal epithelium is highly resistant to pathogen penetration; however, when the epithelium is wounded, the barrier is compromised and pathogens are able to enter the cornea. Therefore, the epithelium tries to restore its protective barrier as quickly and efficiently as possible. It has been found that in order to do so, the cells outside the wound area are signaled to proliferate while the cells inside the wound area are signaled to migrate.(Zieske, et al., 2004) This enables the epithelium to cover the wound area quickly. After re-epithelialization, the epithelium then stratifies to its original 5–7 cell layers. However, resurfacing the wound area does not necessarily mean that the epithelial barrier is restored.

Numerous papers have examined the effects of various factors on wounding and when certain intercellular junctions reappear. All reports agree that the restoration of the barrier lags beyond epithelial wound closure.(Chang, et al., 1996; Kim, et al., 1998; Kim, et al., 1996; Nakamura, et al., 2003; Nejima, et al., 2005; Polunin, et al., 1999; Shimazaki, et al., 1999; Suzuki, et al., 2000; Yi, et al., 2000) However, the length of this lag varies widely between reports. This lag period has been reported to range from a few hours in a cell culture model.(Yi, et al., 2000) to three days for a debridement wound in rats.(Huang, et al., 1990) to two weeks in humans following photorefractive keratectomy (PRK),(Kim, et al., 1998; Kim, et al., 1996) to as long as four weeks in rabbits following PRK,(Chang, et al., 1996) and even to eight weeks following LASIK.(Nejima, et al., 2005) In the current study, a surface biotinylation method was used to assay tight junction integrity. This method makes use of a small compound (EZ-Link Sulfo-NHS-LC-Biotin; Molecular Weight = 556 Da) that normally does not pass through the epithelial tight junctions.(Chen, et al., 1997; Merzdorf, et al., 1998; Xu, et al., 2000) When the junctions are disrupted, the compound passes between cells and into the stroma.(Chen, et al., 1997; Merzdorf, et al., 1998; Xu, et al., 2000; Xu, et al., 2004) This technique allows visualization of the functional epithelial barrier. The current study makes use of this technique to examine disruption and restoration of the barrier function following superficial keratectomy wounds in rats.

2. Materials and Methods

All studies conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Adult Sprague-Dawley rats were anesthetized and a 3-mm superficial keratectomy was performed as previously described.(Hutcheon, et al., 2005; Zieske, et al., 1987) In brief, the central area of the cornea was demarcated with a 3-mm trephine, and rotated gently to cut into the stroma. The circular area was traced with a sharp pair of surgical forceps, and the anterior portion of the stroma was removed by pulling with the forceps. This type of wound leaves a bare stroma with the epithelium and basement membrane removed. The eyes were allowed to heal from 4 hours to 8 weeks. Six rats for each time point had the right eye wounded, and the contralateral eye served as a control. At the time of sacrifice, EZ-Link Sulfo-NHS-LC-Biotin (LC-Biotin; Pierce; Rockford, IL) was applied to all eyes, experimental and control, for 15 minutes. LC-Biotin stably cross-links to proteins, and does not penetrate through intact tight junctions.(Chen, et al., 1997; Merzdorf, et al., 1998) After 15 minutes, the eyes were rinsed with PBS, blotted and enucleated. Eyes were frozen in OCT on dry ice and 6 μ m sections were cut. Indirect immunofluorescence was performed as previously described (Hutcheon, et al., 2005) with anti-laminin (DAKO; Carpinteria, CA), fluorescein-conjugated streptavidin (Jackson ImmunoResearch; West Grove, PA), anti-ZO-1 and anti-occludin (Zymed Laboratories, Invitrogen; Carlsbad, CA). Tissues were coverslipped with Vectashield

mounting media (Vector Laboratories; Burlingame, CA) and viewed and photographed with a TCS-SP2 Confocal Microscope (Leica Microsystems; Heidelberg, Germany).

Determination of healing rates

In initial studies to examine healing rates of superficial keratectomy wounds, corneas were dissected and placed into a completely defined media (Gipson and Anderson, 1980) as previously described. (Zieske and Gipson, 1986) The corneas were pinned to rounded paraffin posts that maintain the corneal curvature. The corneas were allowed to heal for up to 48 hours at 35°C in a 5% CO₂ atmosphere. Healing rates were determined by staining the remaining epithelial defect with Richardson's stain, (Richardson, et al., 1960) photographing the cornea with a Spot camera connected to a Nikon SMZ800 microscope (MicroVideo Instruments; Avon, MA), and measuring the defect area with ImageJ software (National Institutes of Health; Bethesda, MD: <http://rsb.info.nih.gov/ij/>).

3. Results

In an initial set of experiments, the rate of epithelial wound closure over a superficial keratectomy was determined. As seen in Figure 1, an initial lag of 12 hours was seen with wound closure occurring between 36 and 42 hours in an organ culture model. The timing of epithelial wound closure was similar in vivo where 6/6 rats had no apparent epithelial defect at 40 hours, while 6/6 had a small defect at 32 hours. In comparison, a 3-mm debridement wound required 22 hours for epithelial wound closure. (Zieske and Gipson, 1986)

Restoration of barrier function was then determined using LC-Biotin. As seen in Figure 2C₂, LC-Biotin does not penetrate beyond the most superficial layer of epithelial cells in unwounded corneas. Upon wounding, the LC-Biotin penetrated throughout the entire stroma subjacent to the wound area (8 hours; Fig 2A-C₁). At 8 and 24 hours after wounding, the LC-Biotin was seen at the leading edge of migrating epithelium in all cell layers (Fig E₂). The LC-Biotin continued to penetrate all the layers of the epithelium but to decrease in intensity all the way out to the wound edge (Fig A, B, D and E₁). Twenty-four hours post-wounding, LC-Biotin still penetrated through the entire stroma in the center of the wound area (Fig 2D-F); however, in the stroma at the wound edge, where the epithelium migrated to cover the wound, penetration of the LC-Biotin was greatly reduced (Fig 2D). Also, the localization of laminin at the wound edge was patchy and discontinuous (Fig 2D). By 48 hours after wounding (Fig 2G-I), the epithelial defect was closed; however, LC-Biotin still penetrated into the stroma and epithelium in the center of the original wound (Fig 2I). The intensity of the LC-Biotin in the epithelium appeared to decrease with depth of penetration in the wound center. No LC-Biotin was apparent in the epithelium at the wound edge (Fig 2G). Laminin was still localized in a discontinuous pattern at 48 hours (Fig 2G-I). At 72 hours after wounding (Fig 2J-L), no LC-Biotin was observed to penetrate beyond the superficial layer of epithelial cells in 6/6 corneas. Localization of laminin was still patchy compared to unwounded corneas (Fig 2C₂). Continuous laminin localization was not observed until one week post-wounding. (Hutcheon, et al., 2005) No LC-Biotin penetration was observed at longer time points (Data Not Shown).

Occludin in the unwounded cornea was present primarily in the superficial cells (Fig 3F₂). Upon wounding, occludin appeared to be present in all the epithelial cell layers of the leading edge; however, the occludin in the lower layers appeared to be diffuse and disorganized (Fig 3B). This pattern was seen all the way to the wound edge, with no occludin in the basal cell layers (Fig 3A). Outside of the wound area at 8 hours, the expression pattern of occludin was similar to that seen in unwounded cornea (Data Not Shown). At 24 hours, occludin appeared to be in all layers of the epithelium at the leading edge, but appeared to be diffuse and disorganized (Fig 3D). This pattern continued even outside of the wound area; however, as with 8 hours, occludin was not localized in basal cells distal to the leading edge (Fig 3C). By

48 hours, the expression pattern was the same; however, the occludin in the superficial cells appeared to be more organized (Fig 3E). Seventy-two hours post-wounding (Fig 3F₁), occludin was more organized and was approaching the pattern of localization observed in unwounded cornea. However, the occludin continued to be present in more cell layers than unwounded cornea (Fig 3F₂).

In unwounded cornea, ZO-1 was confined mainly to the superficial cell layer of the corneal epithelium (Fig 4A₂). After wounding, there appeared to be no significant change in ZO-1 at the wound edge (Fig 4) or outside of the wound area (Data Not Shown). At the leading edge of 8 hours post-keratectomy (Fig 4A₁), the pattern of ZO-1 localization was similar to that of unwounded (Fig 4A₂) with some faint disorganized ZO-1 in the rest of the epithelial cell layers. By 24 hours (Fig 4B), this disorganized pattern appeared to become more organized, however, still faint. Forty-eight hours post-wounding (Fig 4C), ZO-1 was seen in multiple layers at the center of the original wound area. By 72 hours (Fig 4D), ZO-1 was consistent with unwounded corneal epithelium (Fig 4A₂).

4. Discussion

One of the main functions of the corneal epithelium is to serve as a barrier to the external environment.(Gipson and Sugrue, 1994) Zonula occludens, or tight junctions, encircle the superficial cells of the corneal epithelium, preventing fluid loss and the penetration of potential pathogens.(Sugrue and Zieske, 1997; Wang, et al., 1993; Yi, et al., 2000) Mucins associated with apical cells also play a role in forming the barrier.(Gipson, 2004; Price-Schiavi, et al., 1998) Thus, one of the key roles – perhaps the key role – of epithelial wound repair is to rapidly cover the wound and restore the barrier function.

Our data shows that the functionality of the epithelial barrier is restored 72 hours after wounding – approximately, 24 to 36 hours after the epithelium has resurfaced the wound area. This is in agreement with Suzuki et al.,(Suzuki, et al., 2000) whose studies found that certain intercellular junctions—e-cadherin (adhesion protein of adheren junctions), desmoglein (adhesion protein of desmosomes) and connexin 43 (gap junction protein)—return to normal after a 3-mm PRK by 72 hours post-wounding.(Suzuki, et al., 2000) Interestingly, they found that occludin, an adhesion protein of tight junctions, was present 12 hours post-PRK and was maintained in the migrating cells resurfacing the wound area. According to Suzuki et al.'s results(Suzuki, et al., 2000) and ours, it appears that the mere presence of tight junction proteins is not sufficient to predict the integrity of the barrier function. Rather the subcellular localization must also be determined. Tight junctions are dynamic junctions whose assembly and function are modulated by a number of molecules that stimulate the redistribution of ZO-1 and occludin between the cytoplasm and the cell membrane.(Chen, et al., 2000; De Paiva, et al., 2006)

Our studies made use of LC-Biotin, which stably cross-links to free amine groups present in proteins and other molecules.(Chen, et al., 1997; Merzdorf, et al., 1998; Xu, et al., 2000) Thus, LC-Biotin allows a study of the area of penetration by compounds normally present in the tear film following disruption of the epithelium. As seen in Figure 2, LC-Biotin penetrated through the entire stroma subjacent to the area of epithelial removal. Interestingly, LC-Biotin was not observed in the stroma extending beyond the original wound area, at any time point. This suggests that the epithelial barrier is maintained outside the original wound area. Our results demonstrated that LC-Biotin was observed only between epithelial cells that have migrated to cover the original wound. Disruption of the epithelial barrier was never observed outside the area of the original wound.

In our study, laminin-1 was localized to demarcate the site of the original wound and to examine the extent of basement membrane reassembly. As seen in Figure 2, there appeared to be little

correlation between the reassembly of the basement membrane and the restoration of the barrier function. Our results indicated, that barrier function was restored several days before laminin was localized across the entire wound area. This is somewhat in disagreement with the findings of Suzuki et al.(Suzuki, et al., 2000) who reported that localization of junctional proteins corresponded to the deposition of basement membrane components. However, their report did not examine the functional return of the epithelial barrier.

Although the primary goal of our study was to examine the restoration of the functional barrier of the corneal epithelium, we also examined the localization of two protein components of tight junctions, occludin and ZO-1. Previous studies of these proteins during wound healing have shown that they continue to be expressed during the healing process.(Danjo and Gipson, 1998; Malminen, et al., 2003; Suzuki, et al., 2000) Suzuki et al. observed no changes in occludin expression in stationary or migrating epithelium,(Suzuki, et al., 2000) and Danjo and Gipson reported that ZO-1 expression was diminished only in the first cell of the migrating epithelium in a debridement wound.(Danjo and Gipson, 1998) In a cell culture model, Yi et al.(Yi, et al., 2000) observed that little, if any, change in occludin and ZO-1 occurred in response to lipopolysaccharide challenge, even though the functional barrier was diminished as measured by transepithelial electrical resistance. In our studies, we observed that occludin and ZO-1 localization was altered during wound repair. Our main finding was that both ZO-1 and occludin appeared to be expressed in an increased number of cell layers during the healing process. This was most obvious at the 48 hour time point. This finding is in agreement with that of Wang et al., who observed that ZO-1 was localized in several cell layers when the outermost layer of cells was removed.(Wang, et al., 1993) Similar results have also been observed in healing epidermal wounds.(Malminen, et al., 2003)

While our data and that of others indicates that tight junction integrity is restored within hours or days after wound closure,(Huang, et al., 1990; Yi, et al., 2000) several reports have found that epithelial integrity is still compromised for weeks after refractive surgery.(Chang, et al., 1996; Kim, et al., 1998; Kim, et al., 1996; Nejima, et al., 2005; Polunin, et al., 1999) These studies have primarily used fluorescein penetration as a marker of epithelial integrity. One possible explanation for this discrepancy is that the tight junctions are initially restored, but then disrupted at a later stage of repair. However, our data indicate that LC-Biotin did not penetrate any corneas from time points 3 days to 8 weeks. A second possibility is that fluorescein penetration is the result of altered tear film and/or mucins associated with the ocular surface after PRK or LASIK. This concept is supported by the recent findings of Dursun et al., who found that stripping the epithelium of tear film and mucin 4 lead to a rapid (within 30 seconds) increase in penetrance of fluorescein.(Dursun, et al., 2000) This possibility is also supported by the findings of Nejima et al., who found that LASIK surgery (which has a higher incidence of altered tear film) produces a more prolonged and extensive loss of barrier function as measured by fluorescein uptake.(Nejima, et al., 2005) The concept that cell associated mucins contribute to the epithelial barrier has been recently reviewed by Gipson.(Gipson, 2004) A third possibility is that the differences may be the result of differing chemical properties of fluorescein and LC-Biotin. However, both molecules are negatively charged and similar molecular weights. (Sodium fluorescein 376 Da vs. LC-Biotin 556 Da)

In summary, we have made use of LC-Biotin to observe the disruption and restoration of the epithelial barrier function following superficial keratectomy wounds. This compound does not pass through intact tight junctions and is stably cross-linked to tissues allowing for a comparison between its penetration into the cornea with known tight junction proteins. Our major findings are 1) barrier function is not compromised outside the original wound area, 2) tight junction integrity is restored by 72 hours after wounding (approximately 1.5 days after epithelial wound closure), based on LC-Biotin penetration, 3) barrier function is restored at

the wound edge before the center of the wound, and 4) barrier function is restored before complete reassembly of the basement membrane.

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Figure 1. Graph showing the rate of epithelial wound closure of a 3-mm superficial keratectomy in organ culture. There was an initial lag of about 12 hours, and wound closure occurred between 36 and 42 hours. Values are indicated as mean $\pm$ SEM. At least four corneas were examined per time point.

Figure 2.

Immunolocalization of LC-Biotin demonstrating the restoration of the epithelial barrier function after a 3-mm superficial keratectomy. In unwounded corneas LC-Biotin (Red) did not penetrate beyond the superficial epithelial cell layer (C_2). Upon wounding, LC-Biotin penetrated into the stroma denuded of corneal epithelium (8 hours, A-C). At 24 hours (D-F), LC-Biotin penetrated into the stroma of the remaining wound area; however, the amount of LC-Biotin staining in the stroma at the wound edge was greatly reduced and the localization of laminin (Green) was patchy (D). LC-Biotin penetration was seen through all epithelial cell layers at the leading edge (E_2) with a decrease in intensity towards the wound edge (D and E_1). After epithelial closure (48 hours, G-I), LC-Biotin still penetrated into the stroma at the

center of the original wound (I). No LC-Biotin staining was seen in the stroma at the wound edge (G), and discontinuous and patchy laminin was present in the original wound area (G-I). LC- Biotin was also present in the epithelium at the center of the original wound (I), with the intensity decreasing with depth of epithelial penetration. By 72 hours (J-L), no LC-Biotin penetrated beyond the superficial cells, which was consistent with unwounded cornea (C₂). Laminin localization, on the other hand, continued to be patchy compared to unwounded cornea. Bar = 50 μ m.

Figure 3.

Occludin localization in a 3-mm superficial keratectomy. Eight hours after wounding (A and B), occludin (Green) was present in all the cell layers of the epithelium at the leading edge (B), and became more diffuse in the lower layers of the epithelium. This localization was present out to the wound edge, except that occludin was not observed in the basal layer (A). Outside the wound area (Data Not Shown), occludin localization was similar to that seen in unwounded tissue (F₂). After 24 hours (C and D), occludin was diffuse and disorganized in all the cell layers at the leading edge (D). This pattern continued even outside of the wound area (C). As with 8 hours, there was no occludin present in the basal cell layer away from the leading edge. After wound closure (48 hours; E – wound area), occludin localization was similar to that seen at 24 hours; however, localization in the superficial layer appeared to be more organized. By 72 hours (F – wound area), occludin was similar to unwounded; however, localization still appeared in more layers than with unwounded. Laminin = Red, LC-Biotin = Blue. Bar = 50μm.

Figure 4.

ZO-1 localization in a 3-mm superficial keratectomy. Eight hours after wounding (A_1), ZO-1 localization at the leading edge was similar to unwounded (A_2); however, there was some faint disorganized ZO-1 in the lower layers of the epithelium. By 24 hours (B), the expression pattern of ZO-1 at the leading edge was more organized, but still faint in the lower layers of the epithelium. After wound closure (48 hours; C), organized ZO-1 was seen in multiple layers at the center of the original wound area. By 72 hours (D), ZO-1 localization returned to that seen in unwounded. Outside of the wound area for all time points (Data Not Shown), there was no significant change in expression pattern. Bar = 50 μ m.