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A role for Notch signaling in corneal wound healing

Aihua Ma, MD, PhD¹, Bojun Zhao, MD, PhD², Mike Boulton, PhD³, and Julie Albon, PhD⁴

¹Department of Pediatrics, Provincial Hospital Affiliated to Shandong University, Jinan, China

²Department of Ophthalmology, Provincial Hospital Affiliated to Shandong University, Jinan, China

³Department of Anatomy and Cell Biology, University of Florida, Gainesville, Florida

⁴Cardiff Institute of Tissue Engineering and Repair, Cardiff University, Cardiff, UK

Abstract

To identify the role of the Notch signaling pathway in corneal wound healing, rat corneas receiving either epithelial or stromal wounds were placed in organ culture for up to 3 and 14 days, respectively. Localization of Notch receptors—Notch1, Notch2, and their ligands—Delta1, Jagged1 was determined by immunofluorescence. Wounds were treated with a γ -secretase inhibitor to suppress Notch signaling or recombinant Jagged1 to enhance Notch signaling and morphological changes in the epithelium and stroma were recorded. The expressions of markers of cell proliferation (Ki67) and epithelial differentiation (cytokeratin 3) were assessed by immunohistology. Notch1 and Notch2 were localized to suprabasal epithelial cells in normal corneas. During corneal wound healing, both Notch receptors were detected in suprabasal and superficial epithelial layers. Delta1 and Jagged1 were observed throughout all corneal epithelial cell layers and occasional keratocytes of the stroma in normal and wounded corneas. γ -secretase inhibition of Notch resulted in increased epithelial cell layers, with recombinant Jagged1 activation of Notch leading to a reduction in epithelial cell layers during corneal wound healing. Correspondingly, the activation of Notch resulted in a decreased cytokeratin 3 expression in the corneal epithelium, with no effect on cellular expression of Ki67. Notch signaling pathway suppressed corneal epithelial differentiation during corneal wound healing, but had no effect on epithelial cell proliferation.

The corneal epithelium is a nonkeratinized, mucosal multilayer with a rapid self-renewing capacity. Renewal is essential to maintain a state of dynamic equilibrium with superficial cells being constantly shed and replenished by proliferation, migration, and differentiation of limbal epithelial cells. The complex multilayered structure of the cornea allows it to fulfill its role, namely transparency, refraction, photoprotection, and protection of internal ocular structures from the external environment.¹ The process of corneal wound healing can be divided into several distinct but continuous phases: sliding of superficial cells to cover the denuded surface, cell proliferation, and stratification for re-establishment of multicellular layers. In stromal wound, there are corneal keratocytes proliferation, migration, and differentiation (myofibroblasts or fibromyoblasts)^{2,3} as relevant responses. It is proposed that multiple factors and their interactions are involved in the maintenance of corneal epithelial homeostasis and wound healing.^{4,5} However, the precise mechanisms regulating cell proliferation, differentiation, and stratification have yet to be fully elucidated.

Notch signaling dictates cell fate and critically influences cell proliferation, differentiation, and apoptosis.⁶ The Notch receptor undergoes a ligand-dependent extracellular cleavage that releases the extracellular domain leaving a membrane-tethered intracellular domain.⁷ The subsequent intramembrane cleavage by γ -secretase results in the translocation of the Notch intracellular domain to the nucleus where it influences the decision of cell fate.^{8–12} Jagged and Delta act as transmembrane proteins that interact with Notch receptors.¹³ The Notch signaling pathway controls diverse cellular processes, cell–cell adhesion, and migration events via local cell–cell interactions.^{13–15} Recently, it is reported that Notch2 negatively regulates myofibroblastic differentiation of myoblasts during muscle regeneration following injury.¹⁶

Previously, we have demonstrated that Notch family members including Notch receptors (Notch1 and Notch2) and their ligands (Delta1 and Jagged1) exist in the human corneal epithelium and that Notch signaling is involved in corneal epithelial cell fate decisions in one-dimensional and two-dimensional epithelial cell culture in vitro.¹⁷ The aim in this study was to extend these observations by investigating the importance of the Notch signaling pathway in corneal wound healing.

MATERIALS AND METHODS

Source of tissue

Adult Norwegian Brown rats (6–8 weeks old, mean 7 ± 0.9) were sacrificed (CO_2 and cervical dislocation), eyes were enucleated, and used in the experiments immediately. All protocols in this study conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with permission for research.

Corneal wound healing organ culture

Rat corneal wound healing model was carried out based on the method used by Carrington et al.¹⁸ and Foreman et al.¹⁹ Eyes were cleaned in 2% povidine (Betadine, Seton Healthcare, Oldham, UK), then in triplicate, subjected to one of two types of corneal wounds. Epithelial wounds were created by epithelial removal from a 2 mm trephine marked region in the central cornea using a scalpel blade No. 3. Stromal wounds were achieved using a 2 mm trephine punch to remove one-third depth of rat corneal thickness (approximately 80 μm). Corneo-scleral disks (with approximately 3 mm sclera) were excised from wounded eyes and placed epithelial side down. The corneal concavity of each was filled with molten 1% agar: 1% gelatin (BDH, Poole, UK) mix in serum-free DMEM (Dulbecco's modified Eagle's medium). When the gel had solidified, corneas were inverted and transferred to culture dishes, epithelial-side uppermost. Serum-free Trowell's T8 medium (Invitrogen-Gibco, Paisley, UK) containing 100 $\mu\text{g}/\text{mL}$ streptomycin, 100 $\mu\text{g}/\text{mL}$ kanamycin, 100 U/mL benzyl penicillin, 2.5 $\mu\text{g}/\text{mL}$ fungizone, and 2 mM glutamine was added to each dish to a level just below the limbus and corneas were cultured in a standard incubator at 37 °C. One hundred microliters of Trowels T8 medium was added dropwise onto the surface of the corneal epithelium, twice daily, to ensure that the epithelium remained moist. At required time points, corneas were cryoembedded and sectioned for immunohistology analysis.

Immunolocalization of notch receptors and ligands in rodent corneas during corneal wound healing

Fresh and wounded rat corneo-scleral disks with required time points were excised and rinsed in phosphate-buffered saline (PBS) before being snap frozen in liquid nitrogen-cooled isopentane (BDH). Ten-micrometer cryosections were fixed in acetone (BDH) for 10 minutes, then incubated for 20 minutes in 0.2% Triton X-100 in PBS. The sections were rinsed thrice in PBS, incubated in primary antibody (goat anti-rat Notch 1, Jagged 1, rabbit

anti-rat Notch 2 or Delta 1, Santa Cruz Biotechnology, Clane, UK, all diluted 1 in 100 in PBS) for 2 hours at room temperature. Sections were then washed thrice in PBS. Substitution of primary antibodies with same species serum (goat or rabbit) served as negative controls. Sections were incubated with appropriate Alexa Fluor 488 secondary antibodies (each at 1: 1,000 dilution in PBS, Molecular Probes, Paisley, UK) for 2 hours in the dark, washed with PBS and then mounted in Hydromount (BDH) containing Hoechst 33345 (2 μ L/mL bisbenzimidazole, Sigma, Poole, UK). Images were captured using wide field microscopy (Leica DMRAZ, Wetzlar, Germany) and Leica QFluoro software.

Inhibition and activation of notch signaling during corneal wound healing

γ -Secretase inhibitor ([γ SI] LY L685458, Sigma-Aldrich, Poole, UK) and rat recombinant Jagged1 ([J1] 599-JG, R&D System, Abingdon, UK) as activators were used to evaluate the function of Notch signaling in the regulation of epithelial cell differentiation and proliferation during wound healing. Serum-free Trowell's T8 medium containing either γ SI (25 or 50 μ M) or J1 (5 μ g or 10 μ g/mL) was administered onto the surface of wounded (two wound types, as described above) corneas in triplicate, twice daily. Concentrations of γ SI and J1 were those shown previously to inhibit and activate Notch 1 and Notch 2 in corneal epithelial cells in vitro.¹⁴ Corneas cultured with T8 medium only were as controls. Triplicate corneas were cultured for up to 72 hours for epithelial wounds and 14 days for stromal wounds. At required time points, corneas were cryoembedded and sectioned for corneal morphology analysis.

Corneal morphology

Corneal morphology was assessed in triplicate cryosections of each time point following hematoxylin and eosin staining. In brief, sections were fixed in acetone for 5 minutes, washed in running tap water for 5 minutes before immersing in hematoxylin for 2 minutes. Following further washing in water (5 minutes), sections were incubated in Eosin for 1 minute. Then, sections were washed again and mounted in Hydromount (National Diagnostics, Atlanta, GA). Images were captured using wide-field microscopy (Leica DMRAZ) and Leica QWin software.

Cell proliferation (Ki67) and differentiation (CK3)

Markers of cell proliferation (Ki67) and epithelial differentiation (cytokeratin 3 [CK3]) were localized to corneal sections by indirect immunofluorescence as described above. Sections were dual-labeled by incubation in antibodies against rat CK3 (1 in 100 dilution, AE5, ICN Biomedicals, Costa Mesa, CA) and Ki67 (1 in 50 dilution, clone SP6, Abcam Ltd., Cambridge, UK) for 2 hours. Following washing, sections were then incubated in a solution containing goat anti-mouse Alexa Fluor 488 and goat anti-rabbit Alexa Fluor 594 (Molecular Probes), diluted 1 in 1,000 in PBS.

Sections were washed and mounted in Hydromount containing Hoechst, and images were captured as described above. Ki67 and CK3 immuno-positive cells were counted using Image Pro Plus analysis software (Image Solutions, Bourne End, UK) by a masked observer and expressed as a percentage of total cell number (i.e., Hoechst-labeled nuclei).

Statistical analysis

All experiments were repeated at least three times, and the results were expressed as mean $\pm$ SEM. Statistical analysis was performed using one-way ANOVA or Kruskal–Wallis dependent on distribution of data. $p < 0.05$ was considered statistically significant.

RESULTS

Immunolocalization of Notch1, Notch2, Delta1, and Jagged1 in normal and wounded rat corneas

Expression of the Notch receptors in sections of normal and injured rat cornea was analyzed by immunohistochemistry. In normal adult rat cornea, Notch1 (Figure 1A1–2) and Notch2 (Figure 1B1–2) were localized to subbasal cells in corneal epithelium, with labeling absent in the basal cell layer, uppermost superficial cells, and stromal keratocytes. During corneal wound healing (both types of wounds), Notch1 (Figure 1A3–6) and Notch2 (Figure 1B3–6) were expressed in both corneal superficial and the suprabasal cells.

In unwounded cornea, the expression of Notch ligands Delta1 (Figure 1C1–2) and Jagged1 (Figure 1D1–2) was observed throughout epithelial cell layers in all regions and a few keratocytes (2–10%) within corneal stroma. During epithelial and stromal wound healing, the expression patterns of Delta1 and Jagged1 were similar with normal corneas, which were detected throughout all epithelial cell layers and faintly expressed in the stromal keratocytes.

Corneal morphology after γ SI and J1 treatment

Corneal epithelial wound healing—The epithelium of the unwounded rat cornea comprises five to six layers of cells: a basal columnar layer, three to four layers of suprabasal or wing cells, and one to two layers of flattened superficial epithelial cells. After a central excisional wound with blade, all epithelial cell layers have been removed from the basement membrane. In control corneas receiving no treatment, within 1 hour after a wound, the plasma membrane of basal epithelial cells at the edge of the wound began to flatten and extend pseudopodia onto the basement membrane. Approximately 4 hours after wound, a monolayer apron of basal cells began to migrate onto the wound surface (Figure 2A1). At 24 hours postepithelial wounding, the wound area was thinly covered by epithelial cells (one to two cell layers, Figure 2B1), with no obvious stratification. Complete re-epithelialization, four to five cell layers thick, showing good stratification had occurred by 72 hours (Figure 2C1).

γ SI and J1 had significant effects on the corneal epithelial wound healing. In γ SI-treated (50 μ M) corneas, the leading edge of basal cell started to migrate approximate 4–5 hours after wound (Figure 2A2) and the wounded area was covered by epithelial cells with the beginning of epithelial stratification at 24 hours (Figure 2B2), and increased hypercellularity at 72 hours to seven to eight cell layers (Figures 2C2 and 3A; $p < 0.05$). In J1-treated (10 μ g/mL) corneas, the starting time of basal cell migration was similar with control (Figure 2A3) but epithelial covering of the wound area appeared to be retarded at 24 hours (Figure 2B3), with partial reepithelialization (one to two cell layers thick) and stratification achieved by 72 hours (Figures 2C3 and 3A; $p < 0.05$). Both γ SI and Jagged1 significantly influenced the reepithelialization but not the starting time of cell migration.

Corneal stromal wound healing—Central excisional stromal wounds with trephine in rat corneas led to the removal of all epithelial layers along with a portion of the underlying stroma. After 12 hours (Figure 2D1), at commencement of migration, the leading edge of the epithelium was commonly one cell deep, smoothly increasing in thickness toward the original wound edge with no obvious stratification. The epithelial hypercellularity was obviously observed at the center of the wound at 7 and 14 days postwounding (Figure 2E1 and F1) to a greater extent in γ SI-treated corneas in a dose-dependent manner, with 11–13 epithelial cell layers at 50 μ M γ SI (Figure 2F2) compared with eight to nine cell layers in control corneas at day 14 (Figures 2F1 and 3B; $p < 0.05$). Reepithelialization was slower in

J1-treated corneas in a dose-dependent manner, resulting in a reduced number of epithelial cell layers: two to four layers by day 14 at 10 μ g/mL J1 (Figures 2F3 and 3B; $p < 0.05$).

Cell proliferation and differentiation

Epithelial wound healing—Immunolocalization of Ki67 in epithelial wound appeared to be localized to basal cell layer, but the level of cell proliferation altered following epithelial wounding. After a central epithelial wound, basal epithelial cell proliferation near wound site (peri-center) and limbus was increased (Figure 4A and B). The percentage epithelial cell expression of Ki67 in peri-center (Figure 5A) was from 23% at 0 hours to a peak of 45% at 24 hours, followed by a decline to 22% by 72 hours. In the limbal region, percentage of Ki67 increased from 13% to a peak of 37% between 0 and 24 hours, thereafter decreasing to 14% at 72 hours postwounding (Figure 5B). Cellular expression of Ki67 was not significantly altered in either peri-central or limbal epithelium of γ SI- or J1-treated corneas (Figure 4C–F), at any time points when compared with controls (Figures 4A and B and 5A and B).

The positive immunolabeling of CK3 in rat cornea was detected in all the corneal cell layers following epithelial wounding (Figure 4A), but absent in the limbal basal layer (Figure 4B). As shown in Figure 5C and D, there were no obvious changes of the percentage of CK3 expression in corneal peri-centre (83–90%) and limbus (63–68%) during epithelial wound healing. γ SI had no effect on epithelial cell CK3 expression compared with controls (Figures 4A–D and 5C and D). However, J1 treatment resulted in a significant time and dose-dependent decrease in percentage CK3 cell expression (Figures 4E and F and 5C and D). 5 μ g/mL J1 treatment declined the expression of CK3 from 85 to 10% in peri-center (Figure 5C) and 65–18% in limbus (Figure 5D), whereas 10 μ g/mL J1 caused a decrease expression of CK3 from 90 to 8% and 65–10% in peri-center and limbus, respectively (Figure 5C and D). There was a significant reduction compared with controls ($*p < 0.05$).

Stromal wound healing—During stromal wound healing, there was an increased expression of Ki67 in the peri-center of epithelium (Figure 6A), from 20% to a peak of 50% between 0 and 72 hours, followed by a decline to 19% at 14 days. In the limbal epithelium, the percentage of Ki67 increased from 15% to a peak of 38% between 0 and 72 hours, thereafter decreasing to 13% at 14 days postwounding (Figure 6B). Cellular expression of Ki67 was not significantly altered in either peri-central or limbal epithelium of γ SI- or J1-treated corneas, at any time points when compared with controls (Figure 6A and B). γ SI had no effect on the epithelial cell CK3 expression compared with controls (Figure 6C and D). Nevertheless, 5 or 10 μ g/mL J1 treatment resulted in a significant time and dose-dependent decrease in percentage of CK3 expression throughout the epithelium compared with controls (Figures 6C and D; $*p < 0.05$).

DISCUSSION

After injury to the cornea, the cell cytoskeleton and intercellular junctions are modulated.^{20–23} The initial migrating mass of epithelial cells is generally composed of one to two layers,^{24,25} which migrate until contact inhibition is established by physical contact with adjacent cells. Once the injured surface/corneal stroma has been protected by reepithelialization, the epithelial cell mass re-establishes normal thickness^{26–29} and its more highly differentiated cellular characteristics, allowing the epithelium to return to its steady state. Defining the cellular mechanisms responsible for the differentiation, stratification of corneal epithelial cells is a prerequisite to achieve a complete understanding of corneal wound healing process. A number of growth factors and cell signaling pathways are involved in this process. One possible regulator is Notch signaling, which regulates cell fate

in many different tissues, including the nervous system, vascular system, hematopoietic system, somites, muscle, skin and human corneas, in a wide range of organisms.^{13,17,30}

To investigate the role of Notch signaling in corneal epithelial cell stratification and wound healing, an ex vivo corneal wound healing organ culture model¹⁹ was used to mimic in vivo conditions.

Our results demonstrate that in normal adult rat cornea Notch receptors were only expressed in suprabasal layer. During the process of reepithelialization, their expression appeared in both superficial and suprabasal cells. This change may further bolster the suppression of differentiation of corneal epithelial cells at early stage of corneal wound healing.

There were reports that the Notch1 expression appeared in either limbal basal cells only³¹ or basal and immediate suprabasal cells in both corneal and limbal regions³² which are inconsistent with our study. The discrepancy may be due to the choice of primary antibodies or the particular preservation and treatments of samples that cause alterations of temporal and spatial expression of Notch.

The keratocytes play an important role in corneal wound healing especially for stromal wound. Our present data demonstrated that there is no expression of Notch receptors in keratocytes in either normal or wounded corneas. As Notch mainly exerts its biological function via the interaction with its ligands expressed in adjacent cells, we consider the function of Notch signaling is limited in regulation of stromal keratocytes during corneal wound healing. This is consistent with our observations that γ SI or J1 treatment did not affect the expression of Ki67 in stromal keratocytes, albeit there was an increase of this expression during the stromal wound healing (data are not shown).

Our results demonstrate that in the early stages of corneal wound healing (4 hours in epithelial healing, 12 hours in stromal healing), epithelial cell shape, and number of cell layers at the wound edge were not significantly different from control corneas following Notch inhibition or activation. This is not surprising if Notch signaling is involved in cell differentiation, as the latter occurs in the later stages of wound repair. Following 24 hour epithelial wounding and 72-hour stromal wounding, Notch inhibition significantly promoted epithelial cell stratification and reepithelialization, resulting in increased numbers of cell layers and epithelial cell morphology more typical of the mature (differentiated) epithelium. Activation of Notch signaling, by contrast, lead to a significant repression of cell differentiation (CK3 expression), stratification, and reepithelialization with a reduced number of epithelial cell layers. Our result is in agreement with previous studies that indicate a role for Notch in the inhibition of differentiation in various tissue.^{13,33–35} A report showed Notch1 was down-regulated at early stage of wound and restored at late stage.³² Our results already demonstrated Notch has no effect on proliferation, which happens in early stage of wound, whereas restoration of the Notch expression at late stage may be important to prevent over stratification of corneal epithelial cells and maintain cell fate. It is reported that a Notch1 knockout mouse leads to keratinization in the corneal epithelium.³⁶ Xin et al. found that Notch inhibition by mutation of 14-3-3 σ suppressed corneal epithelial cell differentiation.³⁷ The reason for this seemingly contradictory result may be in part due to the non-Notch-mediated effects of 14-3-3 σ mutation.

γ -Secretase is not the exclusive enzyme of Notch pathway, it also reacts with other substrates, including CD44 and E-cadherin, which are also present in the cornea.^{9,38} Thus, functional changes in corneal wound healing after γ -secretase inhibition in epithelial cells may be a result of factors other than Notch. However, our previous study¹⁷ and a dose-dependent reaction of corneal epithelial stratification to γ SI, combined with the antagonistic

effects of Jagged1 activation on Notch receptors, cell differentiation during corneal wound healing, strongly support a role for Notch signaling in corneal repair.

Wounded corneal treated with γ SI resulted in a significant dose-dependent increase of epithelial cell differentiation (CK3), although there were no changes in epithelial cell proliferation (Ki67) in both epithelial wound healing and stromal wound healing, corroborating the observations that Notch inhibits the corneal epithelial cells stratification and has no effect on proliferation. This is also compatible with our previous studies showed that Notch activation and inhibition did not alter human corneal epithelial cell proliferation in a three-dimensional in vitro model of epithelial cell stratification.¹⁷

To modulate corneal wound healing to achieve better clinical outcomes would benefit many situations, and numerous studies, both in vitro and in vivo, indicate the potential of modulation of cellular signaling pathways and potent growth factors in epithelial or stromal healing.^{18,39–41} The results of this study implicate Notch signaling in the molecular regulation of corneal wound healing. Notch is involved in mediating epithelial cell stratification, including epithelial differentiation and maturation but has no effect on stromal keratocytes during corneal wound healing. Notch signaling appears to control the progression of epithelial cell stratification via suppression of epithelial cell differentiation and may be an important target in therapeutic strategies to manipulate corneal wound repair following trauma or elective surgery.

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Figure 1.

Notch receptors and their ligands in normal and wounded rat corneas. In normal cornea, Notch1 (A1-2) and Notch2 (B1-2) appeared to be expressed in epithelial suprabasal layers, but were absent in uppermost superficial cells, basal cell layer, and stroma. Delta1 (C1-2) and Jagged1 (D1-2) were localized throughout the corneal epithelium and a small number of keratocytes within corneal stroma (red arrow). During corneal wound healing, the expression of Notch1 (A3-6) and Notch2 (B3-6) was in both superficial and suprabasal cells, still was absent from basal cells and stromal keratocytes. Delta1 (C3-6) and Jagged1 (D3-6) were localized throughout all of the epithelium and a small number of keratocytes (red arrow). Blue: Hoechst-stained nuclei. Bar = 20 μ m.

Figure 2.

Hematoxylin and eosin staining of corneal wound healing. Epithelial wound: in control corneas (A1, B1, C1), the epithelium is seen starting the migration onto the wounded surface 4 hours post wounding (A1). At 24 hours post wounding, the wound area was thinly covered by epithelial cells (B1), with complete reepithelialization by 72 hours (C1). In γ -secretase inhibitor (γ SI)-treated corneas (50 μ M), inhibition of Notch did not affect the migration starting time (A2), but the epithelium appeared more stratified in the late stages of wound healing (B2, C2). There were more cell layers with a larger number of flat squamous superficial cells by 72 hours post wounding (C2). In J1-treated corneas (10 μ g/mL), migration began at the same time compared with the control (A3), but reepithelialization was retarded at 24 hours (B3). Less reepithelialization and stratification were observed by 72 hours (C3). Stromal wound: the cell migration began at 12 hours post wounding (D1) following an increase in epithelial cell layer number of five to six at day 7 (E1) up to eight to nine layers by day 14 (F1). In γ -secretase inhibitor (γ SI)-treated corneas (50 μ M), the cell migration began at the same time compared with the control (D2) although cell stratification appeared quicker and to a greater extent than control corneas, reaching 11–13 layers by 14 days (F2). After Jagged1 treated (10 μ g/mL), there was a same starting time of cell migration with control (D3) and a significant decrease in reepithelialization of wounded surface. There was only a formation of two to four cell layers in wounded area by day 14 (F3). Bar = 40 μ m.

Figure 3.

The effect of γ -secretase inhibitor (γ SI) and J1 on corneal epithelial cell layers in corneal (A) epithelial and (B) stromal wounds. A significant dose-dependent increase and decrease in the number of epithelial cell layers at the wound site were identified in γ SI- and J1-treated corneas, respectively, compared with controls (* $p < 0.05$), at all time points following both epithelial and stromal wounds.

Figure 4. Immunolocalization of Ki67 and CK3 in corneal epithelial wound healing at 72 hours. Ki67 expression (red) and CK3 expression (green) were localized to basal cells and suprabasal cells, respectively, in pericentral (A, C, E) and limbal (B, D, F) epithelium. Spatial localization was similar in control (A, B), γ -secretase inhibitor (γ SI) (C, D), and J1 (E, F) treated corneas, although the CK3 expression appeared to be decreased in J1-treated pericentral (E) and limbal (F) corneal epithelium in comparison with the control corneal epithelium. Blue: Hoechst-labeled cell nuclei. Bar = 50 μ m.

Figure 5.

The effect of γ -secretase inhibitor (γ SI) and J1 on epithelial cell proliferation and differentiation in corneal epithelial wound healing. γ SI had no effect on the epithelial expression of Ki67 (A, B) or the CK3 expression (C, D) following wounding, compared with controls. J1 had no effect on the epithelial cell expression of Ki67 (A, B). However, addition of J1 resulted in a significant dose-dependent decrease in the percentage epithelial cell expression of CK3 (C, D) compared with controls (* $p < 0.05$), both in the region adjacent to the wound and at the limbus.

Figure 6.

The effect of γ -secretase inhibitor (γ SI) and J1 on epithelial cell proliferation and differentiation in corneal stromal wound healing. γ SI had no effect on the epithelial expression of Ki67 (A, B) or the CK3 expression (C, D) following wounding, compared with controls. J1 had no effect on the epithelial cell expression of Ki67 (A, B). However, J1 significantly decreased the expression of CK3 (C, D) in a dose-dependent manner compared with controls ($*p < 0.05$), both adjacent to the wound region and limbus.