Corneal Wound Healing Requires IKB kinase β Signaling in Keratocytes

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Abstract

IkB kinase β (IKKβ) is a key signaling kinase for inflammatory responses, but it also plays diverse cell type-specific roles that are not yet fully understood. Here we investigated the role of IKKβ in the cornea using IkkβΔCS mice in which the Ikkβ gene was specifically deleted in the corneal stromal keratocytes. The IkkβΔCS corneas had normal morphology, transparency and thickness; however, they did not heal well from mild alkali burn injury. In contrast to the IkkβF/F corneas that restored transparency in 2 weeks after injury, over 50% of the IkkβΔCS corneas failed to fully recover. They instead developed recurrent haze with increased stromal thickness, severe inflammation and apoptosis. This pathogenesis correlated with sustained myofibroblast transformation with increased α-smooth muscle actin (α-SMA) expression, higher levels of senescence β-Gal activity and scar tissue formation at the late stage of wound healing. In addition, the IkkβΔCS corneas displayed elevated expression of hemo-oxygenase-1 (HO-1), a marker of oxidative stress, and activation of stress signaling pathways with increased JNK, c-Jun and SMAD2/3 phosphorylation. These data suggest that IKKβ in keratocytes is required to repress oxidative stress and attenuate fibrogenesis and senescence in corneal wound healing.

Introduction

IkB kinase β (IKKβ) is a key catalytic subunit of the IKK complex [1]. It plays a crucial role in the activation of NF-κB, which is a transcription factor that binds to κB elements in promoters and enhancers of target genes [2]. Stress stimuli can activate the IKKβ-NF-κB cascade, leading to either activation or repression of gene expression in a highly cell type-specific fashion. In immune cells, i.e. neutrophils and macrophages, this cascade leads to induction of genes coding for cytokines, chemokines, enzymes and molecules with microbicidal activity [3]. The immune cell IKKβ, therefore, plays a crucial role in protection against dangerous environmental stimuli. Although IKKβ is ubiquitously expressed in essentially all mammalian organisms, its role in non-immune cells is less well understood.
With the advent of genetic mutant mice in which the \( Ikk\beta \) gene is deleted in specific cell types, it has become evident that \( Ikk\beta \) has diverse roles in the regulation of homeostasis, stress responses, survival and apoptosis [4]. Studies in mutant mice have shown that the \( Ikk\beta \) is required to maintain homeostasis of the immune responses in skin [5,6], to inhibit sensory excitability in neurons [7], to repress proliferation in hepatocytes [8], and to potentiate apoptosis in mammary epithelial cells, leading to mammary gland involution [9]. The physiological effects of \( Ikk\beta \) could be the consequence of modulation of tissue homeostasis and cell-cell interactions. The intestinal epithelial \( Ikk\beta \), for example, protects the intestinal tract from bacterial infection via the suppression of local inflammation and improvement of epithelial cell survival [10]. Similarly, the hepatocyte \( Ikk\beta \) prevents chemical carcinogenicity by reducing hepatocyte ROS accumulation and apoptosis and alleviating the activation of liver macrophages [11].

The cornea consists of five distinct layers: a stratified non-keratinized epithelial cell layer, the Bowman’s membrane, a highly organized collagenous stroma layer interspersed with keratocytes, the Descemet’s membrane and a single endothelial cell layer [12]. Previously, we used gene knockout approach and investigated the role of \( Ikk\beta \) in corneal epithelial cells [13]. We showed that \( Ikk\beta \) is dispensable for pre- and post-natal corneal epithelium development, but is required for optimal healing of corneal epithelial debridement wounds. Mechanistically, \( Ikk\beta \) is required for activation of the NF-\( \kappa \)B and p38 signaling pathways, which lead to corneal epithelial cell migration for wound healing.

Here we have applied the similar approach to characterize the roles of \( Ikk\beta \) in keratocytes, the residential cells of the corneal stroma. We show that the kerocyte \( Ikk\beta \) is also dispensable for corneal development, but is required for wound healing. In response to mild alkaline burn injury [14], \( Ikk\beta \)-deficient corneas exhibit defective healing associated with excess ROS, stress signaling pathway activation, myofibroblast transformation and senescence. These results suggest that the kerocyte \( Ikk\beta \) modulates multiple stress signaling pathways in corneal wound healing responses.

**Materials and Methods**

**Mouse strains, reagents and antibodies**

The \( Ikk\beta^{F/F} \) mice were a gift from Dr. Michael Karin at the University of California at San Diego and the \( Kera-Cre \) mice were described before [15]. The mice (\( n = 94 \)) used in this work were housed in the Experimental Animal Laboratory at the University of Cincinnati. The procedures carried out in this work are in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol (no. 06-04-19-01) approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Cincinnati. Euthanasia was done by carbon dioxide (CO2) to effect followed by cervical dislocation.

The following antibodies were used in the study: anti-p-SMAD 2 (Ser-465, 467), anti-SMAD 3 (Ser-423, 425) and anti-p-Jun (Ser 63, 73) were from Cell Signaling, anti-\( \alpha \)-SMA from Abcam, anti-\( \beta \)-actin from Sigma-Aldrich, anti-CD45 and anti-CD11b from Invitrogen, anti-p-JNK (Thr-183, Tyr-185) from Promega, and anti-HO-1 from StressGen Biotechnologies.

**In vivo** alkali burn of the cornea

Alkaline burn corneal injuries were done following protocols described before with minor modifications [14]. Briefly, animals were anesthetized by intraperitoneal injection of ketamine hydrochloride (80 mg/kg) and xylazine (10 mg/kg). Ocular surface alkaline burns were produced by...
placing 3MM chromatography paper (Whatman) cut into 2-mm diameter circles previously soaked in 0.05 N NaOH onto the central cornea for exactly 90 seconds. The eyes were continuously washed with PBS for 1 min, terramycin ophthalmic ointment was topically administered to the eyes, and the animals were placed on a warming pad. At least 8 mice were used under each experimental condition.

Evaluation of corneal opacity, histological and immunohistochemical analysis
Corneal opacity was evaluated by stereoscopic microscopy and slit lamp. The opacity was scored as: 0, completely clear cornea, +1, slight opacity, +2, mild opacity with iris and lens visible, +3, severe opacity with iris and lens invisible, but opacity was limited to the cauterized area, +4, extensive opacity throughout the entire cornea. Cryosections of the eye tissues were subjected to H&E staining following standard procedures. Immunohistochemical analysis was done as described previously [13]. TUNEL was done using the ApopTag Plus In Situ Apoptosis Fluorescein Detection Kit in accordance to the manufacturer’s instruction (Millipore). The SA-β-Gal activities were measured at the pH 6.0 using Beta-Glo Assay system (Promega). Stained sections were mounted and photographed using an Axio Observer Inverted Microscope (Carl Zeiss).

Statistical analyses
The data were analyzed by either two-tailed student t-test or ANOVA. * p < 0.05, ** p < 0.01 and *** p < 0.001 were considered statistically significant.

Results
Generation of Ikkβ keratocyte knockout mice
The Kera-Cre transgenic mice carry Cre recombinase gene controlled by the Keratocan promoter [16]. We crossed Ikkβ<sup>F/F</sup> and Kera-Cre mice and identified the Ikkβ<sup>F/F</sup> and Kera-Cre genes in the offspring by genotyping of tail genomic DNA (Fig 1A). To evaluate the efficiency of Ikkβ ablation, we isolated corneal stromal cells from adult eyes, extracted genomic DNA, and performed PCR using primers amplifying the Ikkβ<sup>F</sup> allele. While the products of PCR amplification were detected in cells isolated from Ikkβ<sup>F/F</sup> corneas, they were absent in cells isolated from Ikkβ<sup>F/F</sup>/Kera-Cre corneas, though Gapdh used as control was amplified in both cells (Fig 1B). These data confirmed that IKKβ was successfully ablated in the corneal stroma of Ikkβ<sup>F/F</sup>/Kera-Cre mice, henceforth referred to as Ikkβ<sup>ΔCS</sup>.

Fig 1. Generation of Ikkβ<sup>ΔCS</sup> mice. (A) Schematic illustration of the strategy for generating corneal stroma-specific Ikkβ knockout mice, namely Ikkβ<sup>ΔCS</sup> mice. The Kera-Cre transgenic mice, in which the Cre recombinase gene was controlled by the Keratocan promoter, were crossed with Ikkβ<sup>F/F</sup> mice. In the Kera-Cre/Ikkβ<sup>F</sup>, i.e. Ikkβ<sup>ΔCS</sup> mice, the Ikkβ floxed alleles were ablated specifically in the corneal stromal keratocytes. (B) Genomic DNA of the corneal stroma isolated from the Ikkβ<sup>F</sup> and Ikkβ<sup>ΔCS</sup> mice was genotyped by PCR using primers specific for the Ikkβ<sup>F</sup> allele and Gapdh.

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Normal corneal development of *Ilkβ<sup>ACS</sup>* mice

To assess if the keratocyte IKKβ might be required for corneal development, we used stereoscopic examination of the eyes of *Ilkβ<sup>F</sup>* and *Ilkβ<sup>ACS</sup>* mice from 1 month- up to 8 months-old. The gross morphology and transparency of the eyes of *Ilkβ<sup>ACS</sup>* mice were indistinguishable from those of *Ilkβ<sup>F</sup>* mice (Fig 2A). *Ilkβ<sup>F</sup>* and *Ilkβ<sup>ACS</sup>* adult eyes had also similar corneal thickness as determined by histological examination after H&E staining (Fig 2B). Neither the *Ilkβ<sup>F</sup>* nor the *Ilkβ<sup>ACS</sup>* corneas had obvious evidence of cell proliferation or apoptosis as shown by PCNA staining and Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), respectively. Hence, IKKβ expression in keratocytes is dispensable for corneal development and maintenance.

IKKβ is required for optimal corneal wound healing

To evaluate whether keratocyte IKKβ was required for wound healing after environmental insults [17], we performed mild alkali burn injury on the *Ilkβ<sup>F</sup>* and *Ilkβ<sup>ACS</sup>* corneas and examined healing stereoscopically. Both *Ilkβ<sup>F</sup>* and *Ilkβ<sup>ACS</sup>* eyes displayed haze at 1 day post-injury and gradual resolution of the haze 4–7 days after injury (Fig 3A). However, while all of the *Ilkβ<sup>F</sup>* corneas restored transparency at 2 weeks after injury, approximately half of the *Ilkβ<sup>ACS</sup>* corneas (4 out of 8) had recurrent haze and became cloudy. The opacity score was high at day 0–1 and reduced at 4–14 days after injury in all of the eyes examined. It remained low in *Ilkβ<sup>F</sup>* but became significantly higher in *Ilkβ<sup>ACS</sup>* corneas at 4 weeks after injury (Fig 3B). Histological examination showed that the *Ilkβ<sup>F</sup>* and *Ilkβ<sup>ACS</sup>* corneas were not much different at 1 and 4 days after injury, but at 28 days, though the *Ilkβ<sup>F</sup>* corneas showed normal morphology (data not shown), the cloudy *Ilkβ<sup>ACS</sup>* corneas were swollen with increased stroma thickness (Fig 3C). These *Ilkβ<sup>ACS</sup>* corneas also exhibited severe epithelium disruption, epithelial cell protrusion into stroma and scar tissue formation.

Inflammatory and stress responses in *Ilkβ<sup>ACS</sup>* corneas

Alkali burn injury of the cornea evokes inflammatory responses and cellular stress. Inflammatory responses facilitate tissue remodeling essential for wound healing, but if excessive, they

![Fig 2. Role of IKKβ in corneal development and maintenance.](https://example.com/figure2.png)
obstruct healing and cause severe damage [18]. Given IKKβ’s role in inflammatory signaling, we hypothesized that its ablation in keratocytes would perturb corneal inflammation. The injured corneas were examined by immunohistochemistry using anti-CD11b that detects macrophages and anti-CD45 that detects neutrophils. At the early phase of wound healing, the \( \text{IkB}^{\beta^+} \) and \( \text{IkB}^{\Delta CS} \) corneas were similar, with massive macrophages and neutrophils at 1 day, but no inflammatory cells detected at 4 days after injury (Fig 4A–4C). At the late phase, i.e. 28 days, the \( \text{IkB}^{\beta^+} \) corneas (eye 1 and eye 2) had a few detectable inflammatory cells; however, the opaque \( \text{IkB}^{\Delta CS} \) corneas (eye 1) were filled with macrophages and neutrophils (Fig 4D). On the
other hand, if the IkkβΔCS corneas were transparent (eye 2) at 28 days, they were similar to the IkkβF eyes with little, if any, detectable inflammatory cells. The number of inflammatory cells in IkkβΔCS corneas was more abundant than that in IkkβF corneas (Fig 4E). These observations suggest that IKKβ may prevent corneal opacity by facilitating resolution of inflammation at the late phase of wound healing.

Besides its role in inflammatory signaling, the IKKβ is known to be involved in the modulation of redox homeostasis; its ablation has been linked to severe oxidative stress and tissue injury, which may in turn potentiate inflammation and cytotoxicity [11,19,20]. We therefore examined whether IKKβ ablation affected the stress responses by measuring the expression of hemeoxygenase-1 (HO-1), encoded by an oxidative stress-inducible gene. In contrast to the IkkβF corneas, which did not have any detectable HO-1, the opaque, but not transparent, IkkβΔCS corneas had abundant HO-1 expression at 28 days of injury (Fig 5A and 5B). It is known that elevated oxidative stress can lead to the activation of stress-induced signaling pathways, such as the JNK-c-Jun cascade [21]. Indeed, the expression of HO-1 is accompanied by the activation of stress markers, e.g., p-JNK, p-C-Jun (Fig 5). In addition, the opaque IkkβΔCS corneas had increased phosphorylation of SMAD, markers of TGFβ signaling [22–24]. The IkkβF and transparent IkkβΔCS corneas, in contrast, did not have any detectable p-SMAD. Taken together, the aberrant wound healing responses in the IkkβΔCS corneas correspond to sustained inflammation with concurrent increase of oxidative stress and activation of the stress signaling pathways.

**Cellular activities affected by IKKβ ablation**

TGFβ promotes myofibroblast transformation in corneal wound healing [25,26]. The finding that TGFβ signaling was upregulated in the IkkβΔCS corneas prompted us to examine the expression of α smooth muscle actin (α-SMA), a marker of myofibroblasts. There was indeed abundant α-SMA expression in the opaque IkkβΔCS, but not in the IkkβF and transparent IkkβΔCS corneas at 28 days after injury (Fig 6A and 6B).

Both TGFβ activation and sustained oxidative stress can induce, stabilize and amplify senescence, leading to the detrimental effects of cell death [27,28].
associated β-Galactosidase (SA-β-Gal) [29], although completely undetectable in the IkkβF corneas, was detected in a few cells in the IkkβΔCS corneas at 4 days after injury (Fig 6C). While the SA-β-Gal activity remained undetectable in the IkkβF corneas at 28 days after injury, it was markedly amplified in the opaque IkkβΔCS corneas (Fig 6C and 6D). Concurrently, the opaque, but not transparent IkkβΔCS corneas displayed increased apoptosis, detected by TUNEL staining (Fig 6A and 6B). Our data suggest that IKKβ expression in keratocytes is required for the repression of fibrogenesis, senescence and apoptosis in corneal wound healing.

Discussion

In the present work, we show that IKKβ expression in keratocyte is dispensable for corneal development, but required for optimal wound healing. We and others have previously shown that IKKβ expression in fibroblasts is essential to maintain redox homeostasis, and it does so through NF-κB, which regulates anti-oxidant gene expression [20,30–32]. Data presented here suggest that IKKβ has a similar role in keratocytes, the cornea-specific fibroblasts [17]. IkkβACS corneas exhibit elevated oxidative stress and activation of stress signaling pathways after stromal injury. In contrast to wild type corneas, which eventually recover from mild alkaline burn injury, many of the IkkβACS corneas become cloudy and swollen with scar formation. Our data are consistent with the notion that excessive oxidative stress impede the healing of corneal stromal wounds [33,34].

The corneal keratocytes are quiescent in the absence of external insults, but enter cell cycle and become active under pathologic conditions [35,36]. In response to injury, the keratocytes differentiate to myofibroblasts essential for contraction and wound closure; excessive myofibroblast transformation, on the other hand, will result in fibrosis and scars [37,38]. The IkkβACS corneas have sustained myofibroblast activation defined by the expression of α-SMA, and correspondingly, they exhibit strong activation of the TGFβ pathway, a potent inducer of myofibroblast differentiation [39]. Interestingly, Ikkβ−/− fibroblasts display similar phenotype (Chen, et. al., data not shown). Studies in fibroblasts have shown that loss of IKKβ leads to oxidative stress, which induces c-Jun binding and activation of the TGFβ promoter and gene expression;
TGFβ in turn potentiates myofibroblast transformation and senescence (Chen, et al., data not shown). It is possible that oxidative stress also serves as a molecular link between IKKβ and TGFβ signaling in the IkkβΔCS corneas.

Corneal wound healing involves an early inflammatory phase followed by a late remodeling phase. In the early phase, tissue damage triggers neutrophil infiltration and macrophage invasion. These inflammatory cells produce cytokines, chemokines and molecules with microbiobial activity important for protecting the cornea from infection and environmental insults. Previous studies by Saika, et al. have shown that activation of the IKK-NF-κB pathways in the neutrophil and macrophage makes the major contribution to the inflammatory responses in corneal wound healing [40]. Consistent with this notion, we show that IKKβ in keratocytes is not required for early phase inflammatory responses, but instead seems to be involved in the maintenance of tissue homeostasis. In fact, IKKβ exhibits similar functions in other non-immune cells [41], such as hepatocytes [11], keratinocytes [5] and intestinal epithelial cells [42].

The mechanisms through which IKKβ regulates redox homeostasis and TGFβ signaling have been investigated in fibroblasts. In essence, IKKβ is required for optimal expression of redox scavengers, and IKKβ-null cells have decreased capacity to counteract oxidative stress elicited by environmental insults. When oxidative stress increases to a threshold level, it activates the JNK-c-Jun pathway, which induces TGFβ expression and activity; TGFβ in turn can act through NADH oxidase to further potentiate oxidative stress. Due to activation of the autocrine amplification of the ROS-TGFβ-NOX loop, IKKβ ablation in fibroblasts leads to a progressive increase of oxidative stress and TGFβ signaling, and a gradual myofibroblast transformation and premature senescence (Chen, et. al. unpublished data). It is possible that IKKβ also regulates redox homeostasis in keratocytes, where the activation of the ROS-TGFβ-NOX loop leads to the more severe wound healing defects observed in the IkkβΔCS corneas.

Supporting Information
S1 File. Supplemental file for reviewers.
(PDF)

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Author Contributions
Conceived and designed the experiments: YX. Performed the experiments: LC MM QM. Analyzed the data: LC YX. Contributed reagents/materials/analysis tools: WK QW. Wrote the paper: LC YX.

References


