Important Role of Epiregulin in Inflammatory Responses during Corneal Epithelial Wound Healing

Yuan Zhang, Takeshi Kobayashi, Yasubito Hayashi, Ryuji Yoshioka, Atsushi Shiraisi, Senji Shirasawa, Shibeki Higashiyama and Yuichi Obashi

PURPOSE. To investigate the role played by epiregulin in corneal epithelial wound healing in vivo in epiregulin-knockout (KO) mice and cultured mouse corneal epithelial cells (MCECs).

METHODS. A 2-mm diameter central epithelial wound was created in epiregulin-KO and wild-type (WT) mouse corneas. The size of the unhealed area and the epithelial cell proliferation and migration were examined. Myeloperoxidase assay was performed to determine the number of polymorphonuclear (PMN) cells infiltrating corneal stroma. Real-time PCR was used to determine expression of the mRNA of inflammatory cytokines in the corneal epithelial cells. Expression of chemokine (C-X-C motif) ligand 2 (CXCL2) response to IL-1β was examined in MCECs with or without recombinant mouse epiregulin. Repetitive injuries were created to determine the effect of inflammation in healing in epiregulin-KO mice.

RESULTS. After a single injury, corneal epithelial wound healing and cell migration and proliferation were unimpaired. However, corneal opacities and a larger number of infiltrating PMN cells were observed in epiregulin-KO mice. Expression levels of IL-1β, IL-6, CXCL1, and CXCL2 were higher in epiregulin-KO than in WT corneal epithelia cells. The addition of epiregulin significantly reduced the expression of CXCL2 in response to IL-1β in MCECs. In response to repetitive injuries, a significant delay in healing and more severe opacities were observed in epiregulin-KO mice than in WT mice.

CONCLUSIONS. Our results indicate that during wound healing, epiregulin may regulate the expression of cytokines and chemokines to reduce an excessive accumulation of PMN cells, which will cause corneal opacity and persistent epithelial defects. (Invest Ophthalmol Vis Sci. 2012;53:2414–2423) DOI:10.1167/iovs.11-8869

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The cornea is a transparent tissue which refracts light passing through it. It also acts as a physical barrier to prevent infectious agents from entering the eye. Because the cornea is continuously subjected to various physical, chemical, and biological insults, it is easily injured. The injury can cause various degrees of vision reduction, and thus, proper healing of corneal wounds is essential to maintaining normal corneal functions.

Different kinds of growth factors take part in the repair of corneal wounds. Among these are epidermal growth factor (EGF) family members, such as EGF1 transforming growth factor-α (TGF-α),2 and heparin-binding EGF-like growth factor (HB-EGF),3 which have been extensively studied and shown to play important roles in wound healing.4–6 Epiregulin is a relatively newly discovered member of the EGF family.7 It was purified from the conditioned medium of the mouse fibroblast-derived tumor cell line NIH 3T3/clone T7. Epiregulin has two unique characteristics. First, the distribution of the mRNA for human epiregulin is predominantly expressed in the placenta and peripheral blood leucocytes, whereas other EGF family members are expressed ubiquitously in normal tissues.9 Second, EGF family members exert their biological functions after binding to their receptors, including ErbB1 (EGFR), ErbB2, ErbB3, and ErbB4.10–12 Receptor binding and heterodimerization characteristics of epiregulin are unique among the EGF ligands because epiregulin not only stimulates homodimers of both ErbB1 and ErbB4 but also stimulates all possible heterodimeric ErbB complexes.13–15 These characteristics might enable epiregulin to have nonredundant biological functions that are distinct from other EGF receptors. Findings in keratinocytes also indicated that epiregulin, HB-EGF, amphiregulin, and TGF-α have their own distinct biological functions.16

Topical application of recombinant epiregulin has been reported to enhance the repair rate of murine excisional skin injuries.17,18 On the other hand, Shirasawa et al.19 reported that wound healing of the skin in epiregulin-deficient mice was not impaired in vivo but that these mice developed chronic dermatitis.15 These findings suggested that epiregulin may play a role in the immune and inflammatory responses of keratinocytes.

Inflammatory responses, including infiltration by leukocytes and expression of proinflammatory cytokines and chemokines, are consequences of tissue injury. Appropriate recruitment of leukocytes and cytokine networks are necessary for the healing of injury; however, excessive inflammatory responses prevent healing and finally result in persistent epithelial defects and increased corneal opacities. After corneal injury, preinflammatory cytokines and chemokines such as IL-1β, IL-6, chemokine (C-X-C motif) ligand 1 (CXCL1) or (Gro-1/KC/MSGA-α/NAP-3), and CXCL2 (Gro-2/MIP-2α) are released from innate corneal cells. These molecules are important because they initiate inflammatory responses including the recruitment of immune cells to the injured area.
This study examined the role of epiregulin in corneal epithelial wound healing. To accomplish this, we studied corneal wound healing in epiregulin-knockout (KO) mice and in corneal epithelial cells in culture. We showed that epiregulin is required for corneal wound healing, and it functions by regulating the host’s inflammatory responses.

**Materials and Methods**

**Animals**

Epiregulin-KO mice, on a C57BL/6j background, were created by Shirasawa et al.\(^{20}\) Age- and sex-matched wild-type (WT) C57BL/6j (CLEA, Japan Inc., Tokyo, Japan) were used as controls. Mice at 7 to 9 weeks of age were used in all experiments. Procedures used for all animals conformed to ARVO statement guidelines for the Use of Animals in Ophthalmic and Vision Research.

**In Vivo Corneal Epithelium Wound Healing**

Mice were anesthetized by intraperitoneal injection of 4% chloral hydrate (10 mL/kg), and corneas were anesthetized by topical oxybuprocaine hydrochloride solution (Benoxil 0.4% solution; Santen, Osaka, Japan). The center of the cornea was demarcated by a 2-mm diameter biopsy punch (Kai Industries, Seki, Japan), and corneal epithelial cells in this area were removed with a rust ring remover (Handy Micro Motor; Inami, Tokyo, Japan). After the wound was created, the cornea was covered with ofloxacin ophthalmic ointment (Tarivid; Santen, Osaka, Japan) until animals recovered from the anesthetic. The epithelial cell defect was monitored by fluorescein staining and photography with a fluorescence stereomicroscope (StereO Lumar V12; Carl Zeiss MicroImaging, Tokyo, Japan). The area of unhealed wound was measured in the photographs with AxiosVision version 4.7.2 software (Carl Zeiss MicroImaging).

For repetitive injury experiments, mice were anesthetized at 24, 48, and 72 hours after the initial wounding, and the size of the defect was measured after fluorescein staining. Then, the same 2-mm-diameter wound was created on the same cornea, irrespective of whether the defect area had healed or not. The severity of the corneal damage was determined with the same microscope and photographed with iris reflex illumination (Fiber Imaging FV-2000E; Medical Science, Tokyo, Japan). The severity of the corneal opacity was scored from 0 to 3, that is, 0, no opacity; 1, slight opacity; 2, intermediate opacity; and 3, severe opacity (see Figs. 7B, a). The opacity score was determined by an observer who was masked to the type of treatment.

For bromodeoxyuridine (BrdU) labeling, mice were injected intraperitoneally with 200 µg/g body weight of BrdU (Sigma-Aldrich, St. Louis, MO) 2 hours prior to sacrifice.

**In Vitro Corneal Epithelial Wound Healing**

Cultured mouse corneal epithelial cells (MCECs) were isolated and cultured using an established culture method.\(^{20}\) Cells were cultured in 6-well plates (1 \(\times\) 10\(^5\) cells/well) coated with type I collagen and grown to confluence. After growth factor starvation for 12 hours in CnT-20 (CELLnTec, Bern, Switzerland) medium containing 4 mg/mL insulin (CnT-20/IL), cells were scratched with a 1000-µL micropipette tip to create a linear, constant-width epithelial wound. After the cells that were scraped off were washed out, the cells were refed with CnT-20/IL medium. Cells were photographed immediately and at 12 and 24 hours after the scratching with a phase-contrast microscope (IX-70 model; Olympus, Tokyo, Japan). The remaining acellular area was measured on the photographs by using Adobe Photoshop software (Adobe Systems, San Jose, CA).

**Histology and Immunohistochemistry**

Uninjured eyes and eyes at different hours after a single or repetitive injuries were enucleated and fixed in 4% paraformaldehyde or methanol overnight at 4°C. They were then embedded in paraaffin and 5-µm sections were cut through the central cornea and stained with hematoxylin and eosin. For BrdU staining, slides were exposed to 0.0002% trypsin (Sigma-Aldrich) for 15 minutes, followed by 2 N HCl at 37°C for 30 minutes. Rat anti-mouse epiregulin antibody (1:1000 dilution; R&D Systems, Minneapolis, MN), anti-BrdU antibody (1:1000 dilution; Thermo Scientific), and rabbit anti-mouse polymorphonuclear (PMN) antibody (1:1000 dilution; Cedarlane Laboratories, Ontario, Canada) were used. For the detection of macrophages, 8-µm-thick cryosections were stained with rat anti-mouse F4/80 antibody (1:50 dilution; R&D systems). FITC-labeled anti-rat and anti-rabbit IgG (1:500 dilution; Vector Laboratories, Burlingame, CA) were used as the second antibody. These sections were examined and photographed with a charge-coupled device camera (model DP-50; Olympus) attached to a model BX-50 microscope (Olympus) or an inverted fluorescence microscope (Observer.Z1; Carl Zeiss Micro Imaging).

**Myeloperoxidase Assay**

Myeloperoxidase (MPO) assay was modified and used to determine the number of active PMN cells that infiltrated the cornal stroma after the injury.\(^{21}\) Corneas were excised at 0, 12, and 24 hours after the injury and homogenized in 500 µL of 50 mM phosphate buffer (pH 6.0) containing 0.5% hexadecyl trimethylammonium bromide. Samples were freeze-thawed three times and centrifuged at 10,000g for 15 minutes at 4°C. Then, 20-µL aliquots of the supernatants were added to 80 µL of 50 mM phosphate buffer containing ortho-dianisidine dihydrochloride (16.7 mg/100 mL) and 0.0005% hydrogen peroxide. The change in absorbance at 450 nm was measured continuously for 5 minutes, and the rate of change for each sample was determined. The MPO/cornea units were calculated with a standard curve generated with purified MPO (product number M6908; Sigma-Aldrich). One unit of MPO activity was equivalent to approximately 2 \(\times\) 10\(^5\) PMN cells/ml.

**Assay of Migration of PMN Cells**

Mice were injected intraperitoneally with 2 mL of 5% casein in PBS. Twenty-four hours later, the peritoneal lavage fluid was collected and centrifuged at 1200g for 10 minutes. Then, the collected cells were stained with Vybrant DiO cell-labeling solution (Invitrogen) according to the manufacturer's protocol. Then, 1 \(\times\) 10\(^6\) cells in 300 µL of PBS (pH 7.4) containing 0.5% bovine serum albumin were seeded on the upper side of a FluoroBlok multwall insert system (3-µm-pore size; BD Falcon, San Jose, CA). Cells were preincubated for 30 minutes at 37°C to allow the cells to settle onto the surface of the insert system prior to cell migration assay. Then, 1000 µL of 0.5 µg/mL recombiant mouse (rm)-CXCL2 (R&D Systems) or the vehicle PBS was added to the lower chamber as a chemoattractant, and the cells were incubated for another 90 minutes. PMN cells that migrated toward the lower chamber and adhered to the bottom surface of the wells were photographed with an inverted fluorescence microscope (Observer.Z1; Carl Zeiss Micro Imaging).

**In Vitro Cytokine Induction**

MCECs were cultured in 12-well plates (5 \(\times\) 10\(^4\) cells/well) coated with type I collagen and grown to confluence. Then, the culture medium was replaced with CnT-20/IL for 1 day for growth factor starvation. The cells were then stimulated with 1 ng/mL rm-IL-1β (R&D Systems) for 1 hour with or without preincubation with 0 to 30 ng/mL rm-epiregulin (R&D Systems) for another hour. Then the cells were collected for measurement of CXCL2 expression by quantitative real-time reverse transcription-PCR (RT-PCR) as described below.

**Real-time Quantitative RT-PCR**

Corneal epithelium cells from live mice or cultured MCECs were used. Total RNA was extracted with the RNeasy kit (Qiagen, Valencia, CA) and reverse-transcribed to cDNA, using SuperScript VILO cDNA synthesis kit.
(Invitrogen) according to the manufacturer’s protocol. Real-time RT-PCR was performed as described previously, and the proinflammatory cytokine and chemokine primers used are listed in Table 1. The expression level in the untouched WT corneal epithelial cells was set as 1.0, and the levels of cytokines and chemokines are presented relative to that level. The level of the mRNA for β-actin was used as an internal control.

Statistical Analyses

Data are presented as the means ± SEM. Unpaired t tests and Wilcoxon rank-sum test were used for statistical analysis. A P level of <0.05 was considered significant.

Table 1. Primer Sequences for Real-time RT-PCR

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Results

Up-regulation of Epiregulin during Corneal Epithelial Wound Healing

Epiregulin was not expressed in unwounded corneas (Figs. 1A, a–c), but 16 hours after the epithelial injury, epiregulin was detected in the entire corneal epithelium including the leading edge, the periphery, and the limbus (Figs. 1A, e–g). The level of expression was decreased by 24 hours after the wounding (data not shown).
The level of epiregulin mRNA expressed during epithelial wound healing was determined by real-time RT-PCR. Our findings showed that the mRNA of epiregulin was also significantly up-regulated at 1 hour after the injury (Fig. 1B).

**In Vivo Corneal Epithelial Wound Healing and Cell Proliferation**

To investigate the role of epiregulin in corneal epithelial wound healing in vivo, we created a 2-mm-diameter central corneal epithelial wound in epiregulin-KO and WT corneas (n = 6/group). The unhealed area was measured by fluorescein staining at 12 and 24 hours after the wounding. There was no significant difference in the size of the unhealed area between the epiregulin-KO mice and the WT mice at both 12 and 24 hours after the wounding (Figs. 2A and 2B).

Cell proliferation was determined during corneal epithelial wound healing by a BrdU labeling assay. The number of cells that incorporated BrdU was counted from limbus to limbus after the injury. There was no significant difference in the number of BrdU-positive cells between epiregulin-KO and WT mice (Figs. 2C and 2D).

**In Vitro Corneal Epithelial Wound Healing**

The migration of corneal epithelial cells was determined by using a scratch assay with cultured MCECs from epiregulin-KO and WT mice. Significant differences were not observed at either 12 or 24 hours after the scratch assay between the epiregulin-KO and WT MCECs (Figs. 2E and 2F).
Infiltration of Inflammatory Cells

Although wound healing, cell migration, and proliferation were not impaired in epiregulin-KO mice, the density of the corneal opacity and the number of infiltrating cells were greater after injury in epiregulin-KO mice (Fig. 3A). To determine the kind of cells infiltrating the corneal stroma, sections of the cornea were stained with anti-PMN and anti-F4/80 antibodies. Most of the infiltrating cells in the central stroma were stained with the anti-PMN antibody (Figs. 3B, a and d), whereas cells positive for F4/80 were detected predominantly in the limbal stroma (Figs. 3B, b and e) and rarely in the central stroma (Figs. 3B, c and f) of both epiregulin-KO and WT mice. In addition, the number of PMN cells infiltrating the central stroma was higher in epiregulin-KO than in WT corneas (Fig. 3C).

To determine the number of active PMNs, we used MPO assays of epiregulin-KO and WT corneas (n = 6/group/time point). No MPO activity was detected in uninjured control corneas, but MPO activity was elevated at 12 and 24 hours after injury in both epiregulin-KO and WT corneas. The level of MPO activity was significantly higher in epiregulin-KO corneas than in WT corneas (Fig. 3D).

**Figure 3.** Corneal inflammatory responses after a 2-mm-diameter epithelial wound was created. (A) Representative clinical and histological appearance of the healing corneas after injury in WT and epiregulin-KO mice. Bar, 100 μm. (B) Anti-PMN antibody (a, d) and anti-F4/80 antibody (b and c, limbal cornea; c and f, central cornea) immunohistochemistry staining 24 hours after epithelial injury of corneas of WT and epiregulin-KO mice. Bar, 100 μm. (C) PMN cells were counted in the central stroma of WT and epiregulin-KO corneas (n = 12/group). The number of PMNs in the corneas of epiregulin-KO mice is significantly higher than that in WT corneas. *P < 0.05. (D) MPO activity in wounded corneas. Significantly higher MPO activity can be seen in the corneas of epiregulin-KO mice than in those in WT mice at 12 and 24 hours after injury. Data are expressed as means ± SEM of six mice/group/time point. Time 0 hours represents uninjured corneas. *P < 0.05; **P < 0.01. (E) PMN cell migration assay. PMN cells obtained from WT and epiregulin-KO mice were seeded in the upper chamber of a trans-well plate and chemo-attracted by CXCL2 and vehicle (PBS). DiO-labeled PMN cells that migrated to the lower chamber were photographed. Bar, 200 μm. (F) Numbers of migrated PMNs. Data are means ± SEM. The experiment was repeated three times. NS, not significant.
PMN Migration Assay

PMN cell migration assay was used to determine whether the migration of PMN cells in epiregulin-KO mice was defective. Our results showed no significant difference in the migration ability between WT PMNs and epiregulin-KO PMNs toward both the chemokine CXCL2 and the vehicle (Figs. 3E and 3F).

Expression of Proinflammatory Cytokines and Chemokines during Corneal Epithelial Cell Wound Healing

Expression levels of mRNA of the proinflammatory cytokines IL-1β, IL-6, and TNF-α, the chemokines CXCL2, CXCL1, and CCL2 in corneal epithelial cells were determined by real-time RT-PCR at 2, 6, and 24 hours after a 2-mm-diameter wound was created. Time 0 hour represents unwounded corneas. Data are expressed as means ± SEMs of six mice/group/time point. *P < 0.05; **P < 0.01.

PMN Migration Assay

To determine the molecular basis by which epiregulin regulates the inflammatory responses, we examined alterations in proinflammatory cytokines and chemokines during corneal epithelial cell wound healing.
in the expression of CXCL2, the chemokine most effective in recruiting PMNs in response to proinflammatory cytokine IL-1β, in both epiregulin-KO and WT MCECs. Real-time RT-PCR analysis showed that CXCL2 expression was up-regulated by IL-1β in both epiregulin-KO and WT MCECs and reached a peak at 1 hour after stimulation and then gradually decreased (Fig. 5A).

In WT MCECs, the CXCL2 expression peak was 249–83-fold increased compared to that of control, whereas in

**Figure 6.** Corneal epithelial wound healing after repetitive injury of the cornea in WT and epiregulin-KO mice in vivo. (A) Central epithelial defects 2 mm in diameter were created every 24 hours, and corneas were stained with fluorescein at the first wounding (a and c) and at 24 hours (b and f), 48 hours (c and g), and 72 hours (d and h) after the initial wounding. (B) Measurement of the unhealed area at 24 hours after each wounding. From the second wounding, 48 hours after the initial wounding, healing of the corneas of KO mice was significantly delayed compared to that of WT corneas. Data are means ± SEMs (n = 16/group). *P < 0.05; **P < 0.01.

**Figure 7.** Slit-lamp biomicroscopy photographs of the cornea after a 2-mm-diameter repetitive injury. (A) Corneal epithelium was removed every 24 hours, and the cornea was photographed at 24 hours after each wounding (24, 48, 72, and 96 hours after the initial wound) in WT and epiregulin-KO mice. (B) Evaluation of corneal opacity (a). Photographs of corneal opacity scored from 0 to 3 (b). Opacity scores of corneas in WT and epiregulin-KO mice at 24, 48, 72, and 96 hours after the initial wounding. Data are expressed as means ± SEMs (n = 16/group). *P < 0.05; **P < 0.01.
epiregulin-KO MCECs, it was 726 ± 128-fold increased. The MCECs from epiregulin-KO mice had a significantly higher expression of CXCL2 than the MCECs from WT mice.

**Inhibition by Epiregulin of CXCL2 Expression Induced by IL-1β in MCECs of KO Mice**

To determine whether exogenous epiregulin affects the expression of CXCL2 stimulated by IL-1β, we preincubated epiregulin-KO MCECs with different concentrations of rm-epiregulin for 1 hour before stimulation with IL-1β. Concentrations of 10 and 30 ng/mL rm-epiregulin significantly reduced the expression of CXCL2 by 26% ± 2% and 25% ± 2%, respectively (Fig. 5B).

**In Vivo Corneal Epithelial Wound Healing after Repetitive Injury**

Because an overexpression of inflammatory response delays healing and results in increased scarring,22 we created a repetitive injury model to determine the effect of excessive inflammation in epiregulin-KO mice during wound healing. In epiregulin-KO mice, healing was significantly delayed for the second wound at 48 hours after the initial wounding, with the unhealed area 7.1% ± 2.7% in the epiregulin-KO corneas and 0.7% ± 0.2% in the WT corneas (Figs. 6A, c and g). For the third wound, at 72 hours after the initial wounding, the unhealed area was 14.2% ± 5.6% in the epiregulin-KO corneas and 2.2% ± 0.6% in the WT corneas (Figs. 6A, d and h). For the fourth wound, at 96 hours after the initial wounding, the unhealed area was 13.2% ± 2.6% in the epiregulin-KO corneas and 4.4% ± 0.9% in the WT corneas (Figs. 6A and 6B).

**Biomicroscopic Examinations of Corneas after Repetitive Injuries**

Corneas were examined at 24 hours after each wounding by slit-lamp biomicroscopy. Representative photographs of corneas from epiregulin-KO and WT mice are shown in Figure 7A. There appears to be no difference between the corneal transparency in the unwounded corneas of epiregulin-KO and those of WT mice. However, even though the degree of corneal opacity was increased in both WT and epiregulin-KO mice, higher opacity scores were found in the epiregulin-KO corneas than in the WT corneas (Figs. 7B, b).

**Histopathology after Repetitive Injuries**

Corneas taken at different times after the repetitive injuries were examined by hematoxylin and cosin staining. No obvious structural differences were detected between WT and epiregulin-KO mouse in normal uninjured corneas (Figs. 8A and 8B). After the second wound, at 48 hours after the initial wounding, epithelial cells of WT corneas were resurfaced and had fewer inflammatory cells infiltrating the stroma (Fig. 8C). However in the epiregulin-KO mice, the area of the central corneal defect was infiltrated by many inflammatory cells that accumulated in the stroma (Fig. 8D). In the fourth wound, at 96 hours after the initial wounding, there was a small epithelial defect, and some inflammatory cells had infiltrated the stroma in WT corneas (Fig. 8E), but in epiregulin-KO corneas, the epithelial defect was much larger and many cells had infiltrated the stroma. The resurfaced epithelium of epiregulin-KO corneas lost the normal morphology, which included blood vessels, and seemed to be damaged and loosely attached to the stroma (Fig. 8F). These histological appearances were consistent with the biomicroscopic observations of the corneas.

**FIGURE 8.** Representative histological appearance of healing corneas after repetitive injuries in WT and epiregulin-KO mouse. (A and B) Normal uninjured corneas. (C and D) Corneas after the second wound, 48 hours after the initial wounding. (E and F) Corneas after the fourth wound, 96 hours after the initial wounding. More infiltrating cells can be seen in the stroma of KO mice than in WT stroma after the injury. Arrowheads, blood vessels; arrow, leading edge of the wound. Bar, 200 μm.

**DISCUSSION**

Our results showed that wound healing was not delayed in the epiregulin-KO corneas after a single epithelial injury in vivo. However, an excessive inflammatory response predominantly by the infiltration of PMN cells into corneal stroma was demonstrated. This might have been due to increased expression of proinflammatory cytokines and chemokines released by corneal epithelial cells. In addition, we demonstrated the inhibitory role of epiregulin in CXCL2 expression induced by IL-1β. As a result of the excessive inflammatory response, we found that healing was delayed and was accompanied by more severe corneal opacity after repetitive injuries in KO mice. These findings demonstrated the important role of epiregulin in inflammatory response during corneal epithelial wound healing.

Epiregulin is a relatively new member of the EGF family, and it plays important roles during wound healing. We found that wound healing, cell proliferation, and cell migration were not impaired in epiregulin-KO mice in vivo. The absence of a change in cell proliferation or migration might be due to a compensation by other EGF family members induced after the injury (see Supplementary Material and Supplementary Fig. S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11–7941/-DCSupplemental). These findings suggest that epiregulin may not play a crucial role in either epithelial cell migration or proliferation during wound healing in vivo. However, epiregulin-KO mice had a greater number of PMN cells that had infiltrated the central stroma and an increase in the expression of proinflammatory cytokines and chemokines than WT mice. These results suggest that epiregulin probably plays a role in the inflammatory responses during corneal wound healing.

Studies have demonstrated that anterior keratocytes undergo apoptosis after corneal epithelial injury.23 In this study, apoptosis of keratocytes was detected by TUNEL assay 24 hours after the injury in both epiregulin-KO and WT corneas. Although epiregulin-KO corneas had more apoptotic cells in the stroma than WT corneas (see Supplementary Figs. 2, a and d, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11–7941/-DC), the apoptotic cells appeared to be the infiltrating PMN cells. In addition, the results of immunostaining of keratocan, which is secreted by keratocytes, was not significantly different between the epiregulin-KO and WT corneas (see Supplementary Figs. 2, b and e, http://www.iovs.org/lookup/suppl/doi:10.
Sections were also immunostained with anti-zSMA antibody to detect fibroblasts and myofibroblasts after the injury, but no positive staining was found in either epiregulin-KO or WT corneas (see Supplementary Figs. 2, c and f, http://www.iovs.orglookup/suppl/doi:10.1167/iovs.11–7941/-/DCSupplemental). However, the apoptosis of keratocytes and the presence of fibroblasts and myofibroblasts after repetitive injury need to be investigated in greater detail in further studies.

To determine the mechanism for the enhanced recruitment of PMN cells into epiregulin-KO corneas, we examined the ability of PMN cells to migrate. However, results showed no significant difference in the migration ability of PMN cells obtained from epiregulin-KO and WT mice.

Investigators have suggested that different inflammatory cytokines and chemokines are the key agents that control the initiation and regulation of inflammatory events. In inflamed human corneas, CXCL1, CCL2, and IL-8 are highly expressed and contribute to the recruitment of inflammatory cells. We found an up-regulation of IL-1β, IL-6, CXCL1, and CCL2 after the injury, and the elevation was significantly higher in the epiregulin-KO corneal epithelium than in the WT corneal epithelium.

IL-1 is produced constitutively by untouched corneal epithelial cells and is released upon injury. It is an important regulator of ocular surface inflammatory responses. For example, after inhibiting the elevated IL-1β by anti-IL-1β antibody in Pseudomonas aeruginosainduced ocular infections, the degree of corneal inflammation and rate of perforation were reduced by a reduction in the CXCL2 level and the number of PMN cells. Topical application of IL-1 receptor antagonist inhibited inflammatory cells from infiltrating the cornea after epithelial injury. IL-1 can also stimulate the production of other inflammatory mediators. Thus, Yang et al. reported that CXCL1, IL-6, and IL-8 (of the 36 molecules tested) were elevated after exposure to IL-1β in Hela cells. Narayanan et al. reported similar results in human corneal epithelial cells. IL-6, on the other hand, is a pleiotropic cytokine which has been reported to promote corneal inflammation by inducing the production of CXCL2 and CCL3 after simplex virus-1 infection. CXCL1 and CCL2 are chemottractants for PMNs. Mouse CXCL2 is the homologue of human chemokine IL-8 and is the predominant chemottractant for PMNs. It has been reported that a down-regulation of CXCL2 resulted in a reduction of the severity of corneal inflammation. Our results showed that severe inflammatory responses accompanied by dense corneal opacities were present in epiregulin-KO mice. These findings might be explained by the elevated expression of the proinflammatory cytokines IL-1β and IL-6 and the chemokines CXCL1 and CCL2.

CCL2 is a chemottractant for macrophages. After corneal cauterization in both CCL2- and CCR2-KO mice, the number of macrophage infiltrating the cornea was reduced, but the number of PMN cells and the degree of corneal opacification and edema were the same as those in control mice. In our study, the expression of CCL2 was increased after injury, but the difference between the epiregulin-KO and WT mice was not significant. Together with the results of the immunohistochemistry studies, our findings indicate that the abnormal inflammatory response after corneal epithelial injury in epiregulin-KO mice was probably due to an overinfiltration of PMN cells rather than macrophages.

The proinflammatory cytokine IL-1β is released after injury and mediates the infiltration of PMN cells into the inflamed site through the regulation of CXCL2. To determine the molecular mechanism of how epiregulin regulates the inflammatory responses during corneal wound healing, we investigated the expression of CXCL2 in response to IL-1β in epiregulin-KO and WT MCECs. MCECs from epiregulin-KO mice had significantly higher induction of CXCL2 than MCECs from WT mice. This suggests that epiregulin might contribute to inflammation after injury through regulation of the expression of CXCL2 and the consequent PMN cell infiltration.

To investigate the effect of exogenous epiregulin, rm-epiregulin was added to the culture medium of MCECs from epiregulin-KO mice before stimulation by IL-1β. As expected, the expression of CXCL2 was significantly reduced by rm-epiregulin. Although epiregulin partially inhibited CXCL2 expression, this appears to be one of the mechanisms by which epiregulin regulates the inflammatory response during corneal wound healing.

PMN cells are considered the cells most destructive to corneal tissues during inflammation, and they not only eliminate microbial pathogens and secrete re-epithelialization cytokines (e.g., IL-1, IL-6, and TNF-α). However, they also release various proteases that can destroy tissues. It has been reported that corneal epithelial wound healing is significantly delayed by PMN cells and PMN lysates from an organ culture system.

Because an overinfiltration of PMN cells was found after injury in the epiregulin-KO mice, we hypothesized that the wound healing should be delayed because of the excessive PMN cells. To demonstrate this, we created a repetitive epithelial injury model. As expected, healing was significantly delayed in epiregulin-KO mice from the second injury, accompanied by more severe corneal opacity, than in WT mice. The expression of CXCL2 was also significantly higher after repetitive injury in the epiregulin-KO mice than in WT mice (see Supplementary Fig. S3, http://www.iovs.orglookup/suppl/doi:10.1167/iovs.11–7941/-/DCSupplemental). After the fourth wound, 96 hours after the initial wound, blood vessels were observed in the corneas of epiregulin-KO mice. Macrophages have been reported to stimulate angiogenesis and phagocytosis of apoptotic PMN cells during wound healing, in addition to producing a large number of inflammatory mediators. In our study, F4/80-positive macrophages were detected mainly in the limbal stroma, and no obvious difference was found between epiregulin-KO and WT corneas. But the recruitment and the role of macrophages at a later stage of wound healing, after repetitive injury, need to be investigated further.

It is believed that EGRF regulates multiple facets of wound healing, including inflammation, wound contraction, migration, proliferation, and angiogenesis. Repertinger et al. reported that EGRF-null mice had delayed wound closure and an intense infiltration of inflammatory cells especially PMN cells into cutaneous incisions. In our study, we found a new role for epiregulin, one of the EGRF ligands, in the inflammatory responses during corneal epithelial wound healing. Our results suggest that epiregulin should contribute to more effective treatments of corneal inflammatory diseases.

References

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