Corneal Wound Healing, a Newly Identified Function of CAP37, Is Mediated by Protein Kinase C Delta (PKC δ)

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PURPOSE. The neutrophil-derived granular protein, CAP37, an innate immune system molecule, has antibiotic and immunomodulatory effects on host cells, including corneal epithelial cells. We previously showed that CAP37 modulates corneal epithelial cell migration, adhesion, and proliferation, and that protein kinase C delta (PKC δ) mediates CAP37-induced chemotaxis of these cells. The objective of this study was to investigate the hypothesis that CAP37 facilitates corneal wound healing through the PKC signaling pathway.

METHODS. The standard "scratch" assay performed on monolayers of corneal epithelial cells was used to measure the in vitro effect of CAP37 on wound closure. In vivo wound healing in response to CAP37 was measured using a mouse corneal epithelium abrasion model. In vitro and in vivo wound closure were monitored over 48 hours. The PKC δ was visualized during wound closure in cell monolayers and corneal epithelium by immunohistochemistry. The importance of PKC δ in CAP37-induced corneal wound healing was assessed by siRNA.

RESULTS. We found that CAP37 accelerated wound closure in vitro and in vivo. Maximal closure occurred with concentrations of CAP37 between 250 and 500 ng/mL. Topical applications on mouse cornea led to re-epithelialization of the cornea by 24 hours. Immunohistochemistry of in vitro and in vivo wounds revealed a local increase of PKC δ along the wound edge in CAP37-treated cell monolayers and corneas, compared to untreated controls. CAP37-induced corneal wound healing was significantly reduced in vivo upon treatment with PKC δ siRNA.

CONCLUSIONS. These findings support the hypothesis that CAP37 facilitates corneal wound healing through the activation of PKC δ .

Keywords: antimicrobial proteins, corneal epithelium, corneal wound healing, protein kinase C

During corneal epithelial wound healing, essential cellular processes, including proliferation, differentiation, migration, and adhesion of corneal epithelial cells to the basement membrane, must occur rapidly to prevent vision loss or impairment.¹ The corneal epithelium is comprised of three layers of cells, the outer squamous cell layer, the middle wing cell layer, and the inner columnar or basal cell layer. The basal epithelial cells are the only cells capable of proliferating.² When the corneal epithelium is damaged, basal cells proliferate and begin to migrate across the lesion to cover it completely. Then, the cells differentiate into wing cells before they finally become part of the squamous cell layer.³⁻⁵ These processes are facilitated by a number of growth factors, including but not limited to epidermal growth factor (EGF), hepatocyte growth factor (HGF), TGF- β , and platelet derived growth factor-BB (PDGF-BB).¹ While these growth factors are well-established players in normal corneal wound healing, there is accumulating evidence that other proteins, such as CAP37, found in immune cells and the tear film are important contributors to the overall process, thereby ensuring complete fidelity and integrity of the wound. Identifying the role of CAP37 and CAP37 signaling

mechanisms involved in corneal wound healing is the focus of this study.

Previous studies from our laboratory have shown that CAP37, an antimicrobial protein constitutively expressed in the azurophilic granules of human neutrophils,⁶ has potent regulatory effects on host cells.⁷⁻¹¹ It is an effective regulator of monocytes,⁸ macrophages,⁹ and microglia,¹¹ and also regulates endothelial¹² and smooth muscle cell⁷ functions. Furthermore, CAP37 has profound immunomodulatory effects on human corneal epithelial cells (HCECs).¹⁰ CAP37 promotes HCEC proliferation and migration, and upregulates adhesion molecules on HCECs that are important in leukocyte-endothelial interactions as well as epithelial-extracellular matrix interactions.^{10,13} Our findings from the well-characterized rabbit model of *Staphylococcus aureus* keratitis demonstrated that CAP37 was expressed not only in the granules of migrating neutrophils as expected, but also in corneal epithelial cells.¹³ Corneal epithelial expression of CAP37 was observed as early as 5 hours after infection and occurred before the influx of neutrophils that entered the cornea at approximately 15 hours.¹³ Others have reported that CAP37 can be measured in human nasolacrimal ducts.¹⁴ The in vivo expression of

CAP37 in the cornea and its effects on corneal epithelial cells in vitro suggest that it could serve as an important regulator of inflammation and corneal wound healing. In a previous study, we used a combination of technical approaches, including the use of pharmacological inhibitors, siRNA, immunodetection, and kinase activity assay, to demonstrate that CAP37 mediates HCEC migration through the activation of a G protein-coupled receptor and PKC δ .¹⁵ Since cell migration, adhesion, and proliferation are essential processes in wound healing, we undertook the current study to investigate the ability of CAP37 to facilitate corneal wound healing using in vitro and in vivo techniques. Immunohistochemical and siRNA techniques were used to determine if PKC δ is activated by CAP37 and mediates corneal wound healing.

MATERIALS AND METHODS

Cell Culture

The SV40 adenovirus immortalized HCECs were obtained from Dr James Chodosh (Boston, MA, USA). The HCECs were maintained as described previously^{10,15} in defined keratinocyte serum-free media (KSFM; Gibco, Grand Island, NY, USA) supplemented with L-glutamine (2 mM; Gibco), antibiotic-antimycotic (0.1 units/mL penicillin G sodium, 100 μ g/mL streptomycin sulfate, 0.25 μ g/mL amphotericin B; Gibco), and growth supplements as provided by the manufacturer. The HCECs used in these experiments were between passages 10 and 20.

Primary HCECs were isolated from donor corneas acquired from the Lions Eye Bank (Oklahoma City, OK, USA) and cultured as described previously.¹⁵

Production of Recombinant CAP37

Recombinant CAP37 (rCAP37) was produced in human embryonic kidney (HEK) 293 cells using an RSV-PL4 expression vector.¹⁶ The recombinant protein was purified on an HPC4 immunoaffinity column as described previously.¹⁷ All preparations of rCAP37 were dialyzed in 0.01% acetic acid and determined to be pure by SDS-PAGE and Western blot analysis. Functional activity was assessed using the modified Boyden chemotaxis chamber assay as published previously.^{10,15} The rCAP37 preparations used in these studies had < 0.05 endotoxin units/ μ g of protein as determined by the limulus amebocyte lysate assay (QCL 1000; Lonza, Basel, Switzerland).

Animals

The C57BL/6J female mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were acclimated for 4 to 7 days and were 8 weeks of age at the start of experiments. All animals were maintained and handled according to institutional guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

In Vitro Model of Wound Healing

An in vitro scratch assay was used to determine the ability of rCAP37 to facilitate corneal wound healing. Human corneal epithelial cells were cultured as described above until they reached a confluent monolayer. Each monolayer was scratched using a 10 μ L pipette tip to create two perpendicular lines. Monolayers were treated with heparin binding-epidermal growth factor (HB-EGF, 250 ng/mL; Becton Dickinson, Franklin Lakes, NJ, USA), rCAP37 (25–2000 ng/mL), or KSFM without growth supplements (KSFM basal medium; Gibco). Wound closure was monitored at 0, 18, 24, and 48

hours using a camera-equipped inverted microscope (TE2000-E; Nikon, Melville, NY, USA). Time-lapse images of in vitro wound closure were obtained using a camera-equipped inverted microscope (TE2000-E; Nikon) from 0 to 18 hours. Quantitation of wound closure was determined using ImageJ software (National Institutes of Health [NIH], Bethesda, MD, USA). Results are presented as the percentage of wound closure.

In Vivo Model of Wound Healing

Mice were anesthetized using ketamine (100 mg/kg; Bionichepharma, LLC., St. Lake Forrest, IL, USA) and xylazine (10 mg/kg; Rompun; Bayer Corp., Shawnee Mission, KS, USA), and the right cornea was wounded as follows. A disposable biopsy punch (2 mm; Miltex, York, PA, USA) was used to demarcate the mouse cornea. The corneal epithelium was carefully removed within the 2 mm demarcated area with a 0.5 mm burr using the AlgerbrushII (Alger Company, Inc., Lago Vista, TX, USA). The corneal abrasions were treated at 0 and 16 hours with HB-EGF (250 ng/mL), rCAP37 (250 ng/mL), or vehicle control (0.9% sodium chloride, pH 5.5; Baxter, Deerfield, IL, USA). Corneal abrasions were visualized using sterile fluorescein sodium ophthalmic strips USP (Fluorets, Chauvin Laboratory, Aubenas, France) dampened with sterile PBS. Images were taken at 0, 16, 24, and 48 hours immediately following fluorescein staining using a surgical microscope equipped with a camera (Carl Zeiss OPMI VISU 140; Carl Zeiss Surgical, Inc., Oberkochen, Germany). The areas of the open wound were quantitated using ImageJ software (NIH) and the measured area of each wound was expressed as a percentage of the starting area for this particular wound. This was the most accurate assessment because a small variability existed between the starting size of each wound. The normalized results then were reported as percent of wound closure.

Histology

Whole mouse eyes were collected for histology at 0, 6, 16, 24, and 48 hours after wounding and were immediately placed in Prefer fixative (Aantech LTD., Battle Creek, MI, USA) for 20 minutes before being transferred to 70% ethanol. Tissues were paraffin-embedded and cut at a thickness of 5 μ m, mounted on SuperfrostPlus slides (Statlab Medical Products, Lewisville, TX, USA), and subsequently deparaffinized, rehydrated, and washed in deionized water. Sections were stained with hematoxylin (Leica Microsolutions, Buffalo Grove, IL, USA) and rinsed twice in deionized water before being washed in Blue Buffer (Leica Microsolutions). The sections finally were washed in deionized water and 95% ethanol before being counter stained with eosin (Leica Microsolutions). Sections were dehydrated in ethanol and cleared in xylene.

Immunohistochemistry

Sections were blocked for 30 minutes in Rodent Blocker M (BioCare Medical, Concord, CA, USA), washed in deionized water, and blocked in peroxide block (Cell Marque, Rocklin, CA, USA) for 10 minutes. Sections then were washed and incubated overnight at 4°C with rabbit anti-PKC δ (4 μ g/mL; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Sections incubated with 4 μ g/mL rabbit IgG (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C served as controls for nonspecific staining. After incubation with primary antibody, sections were washed in tris-buffered saline (TBS) and incubated with rabbit-on-rodent horseradish peroxidase (HRP)-polymer (BioCare Medical) for 30 minutes. Following

three washes in TBS, sections were stained with 3', 3'-diaminobenzidine tetrahydrochloride (DAB) chromogen (Cell Marque), washed in deionized water, and counterstained with Immuno Master Hematoxylin (American Master[®]Tech Scientific, Inc., Lodi, CA, USA). Images of the stained tissues were obtained using an inverted microscope (TE2000-E; Nikon) equipped with a camera.

Immunofluorescence

The HCECs and primary HCECs were cultured on LabTek II glass chamber slides (Nunc, Rochester, NY, USA) and starved overnight in basal KSFM (Gibco). For wounding studies, monolayers were scratched with a 10 μ L pipette tip or left unscratched and treated with 1 μ M phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, St Louis, MO, USA), rCAP37 (25–500 ng/mL), or basal KSFM (Gibco) for 15 minutes. After treatment, the cells were fixed and treated as described previously¹⁵ using mouse anti-PKC δ (Santa Cruz Biotechnology), mouse anti-PKC θ and anti-PKC α antibodies (Becton Dickinson), and mouse IgG control (Jackson ImmunoResearch, West Grove, PA, USA) to detect the PKC isoforms PKC δ , PKC θ , and PKC α . Images were obtained using an inverted epifluorescent microscope (TE2000-E; Nikon).

siRNA Transfection and Gene Silencing

Stealth RNAi (10 μ M; Ambion, Grand Island, NY, USA) directed against PKC δ or Stealth RNAi siRNA Negative Control Hi GC (10 μ M, Ambion) was delivered into the mouse conjunctiva through a 5 μ L subconjunctival injection using a 33-gauge needle (Hamilton, Reno, NV, USA). After subconjunctival injection, the corneas were wounded immediately using the AlgerbrushII and treated as described above. Wound closure was quantitated at 16 and 24 hours as described. Animals were humanely euthanized at 24 hours and each cornea was excised using a SklarSafe Safety Scalpel #11 (Sklar, West Chester, PA, USA). Tissues were immediately flash frozen in liquid nitrogen. Corneal homogenates were prepared as described below and analyzed for levels of PKC δ by Western blot analysis to confirm the efficiency of each knockdown. The level of PKC δ in the knockdown cornea was compared to the Stealth RNAi siRNA Negative Control Hi GC (Ambion).

Protein Extraction and Western Blotting

Mouse corneas were excised using a SklarSafe Safety Scalpel #11 (Sklar) and frozen at the indicated time points as described above. Corneas were placed in 200 μ L of radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific, Waltham, MA, USA) containing a 1X cocktail of cOmplete ULTRA Protease Inhibitors (Roche, Indianapolis, IN, USA). Tissue homogenates were created by disrupting the corneas for 10 minutes at maximum speed in the Bullet Blender (Next Advance, Inc., Averill Park, NY, USA) using 0.9 to 2 mm stainless steel beads (Next Advance, Inc.). The homogenates were centrifuged at 16,000g for 10 minutes and the protein concentration of the supernatant from each corneal homogenate sample was determined using a BCA protein concentration assay (Thermo Fisher Scientific).

Protein (20 μ g) from each sample of corneal homogenate was analyzed by electrophoresis on a 10% SDS-PAGE gel and transferred to nitrocellulose membranes (Whatman, Inc., Florham Park, NJ, USA) for Western blot analysis. Blots were treated as described previously to quantify PKC δ and β -actin.¹⁵ Blots were analyzed and semiquantitated using ImageJ software (NIH).

Statistics

In vitro wound healing experiments were analyzed using a 1-way ANOVA followed by a Dunnett's multiple comparison test. In vivo wound healing and PKC δ knockdown studies were analyzed using an unpaired *t*-test. GraphPad Prism 4.03 (GraphPad Software, Inc., San Diego, CA, USA) was used for statistical analysis. The independent means of experimental values are shown $\pm$ SEM. *P* < 0.05 was considered significant for all statistical analyses.

RESULTS

CAP37 Facilitates Wound Closure In Vitro

Previous studies have shown that CAP37 mediates HCEC proliferation, migration, and adhesion,¹⁰ leading us to hypothesize that CAP37 may facilitate the process of corneal wound healing. To investigate this hypothesis, an in vitro scratch model was used. Our findings showed that CAP37 promoted wound closure in vitro in a dose-dependent manner (Fig. 1A). CAP37 maximally facilitated wound closure when used at concentrations of 250 to 500 ng/mL. The percentage of wound closure in CAP37-treated wounds with 250 ng/mL of CAP37 was almost 71% at 48 hours and was significantly greater (*P* < 0.001) than basal media-treated samples that showed approximately 41% closure. Treatment of wounds with 500 ng/mL of CAP37 resulted in approximately 62% closure and was significantly greater than the buffer control (*P* < 0.05). HB-EGF, which was used as the positive control, showed almost 88% closure of treated monolayers (Fig. 1A). Representative images of the in vitro scratch assay taken at each time point show the extent of closure in response to the various treatments. Wounds treated with HB-EGF were closed completely by 24 hours, whereas wounds treated with 250 ng/mL of CAP37 required between 24 to 48 hours for complete closure to occur. Buffer-treated wounds did not reach full closure even after 48 hours of treatment (Fig. 1B).

Time-lapse microscopy studies of in vitro wound closure during the first 18 hours after wounding revealed differences in the manner in which the leading edge of the cells responded to HB-EGF and to CAP37 (see Supplementary Videos S1, S2, and S3). In the CAP37-treated samples, some individual cells detach from the leading edge and crawl rapidly across the wounded area, independently of the other cells. The cells showed polarization, with obvious lamellipodia indicative of activation. Not all cells at the leading edge showed activation or produced lamellipodia. However, the edge of the wound displayed more dynamic activity than the HB-EGF-treated monolayers. The HB-EGF-treated cells showed a strikingly different method of wound closure. These cells appeared to advance in a sheet and individual cells did not detach or appear to migrate independently of the advancing sheet of cells across the wound. Morphological changes seen in the CAP37-treated cells, such as polarization and lamellipodia formation, were not as apparent in the HB-EGF or basal KSFM-treated monolayers (see Supplementary Videos S1 and S2). Our experiments could not discern the extent to which migration and/or proliferation contributed to closure of the wound with each of these mediators. However, the classic bell-shaped curve associated with chemotactic agents is observed with CAP37 treatment in Figure 1A, suggesting that CAP37-induced migration may be critical to the wound closure. Additional studies (data not shown) using the Click-iT Plus EdU kit (Molecular Probes, Grand Island, NY, USA) to address the contribution of proliferation to CAP37-mediated wound closure indicated that at the optimal concentration of CAP37 (250 ng/mL) and the

FIGURE 1. The protein CAP37 promotes wound closure in an in vitro scratch model. **(A)** The HCEC monolayers were grown to confluency and scratched using a 10 μ L pipette tip. Scratched HCEC monolayers were treated with HB-EGF (250 ng/mL), rCAP37 (25–2000 ng/mL), or were left untreated in basal KSFM. Wound closure was monitored at 0, 18, 24, and 48 hours using a camera-equipped inverted microscope. The histogram represents data acquired at 48 hours and the values are the mean $\pm$ SEM of the percentage of wound closure. The data are representative of at least 4 independent experiments. The percentage of wound closure in treated monolayers was compared to untreated controls by 1-way ANOVA followed by Dunnett's multiple comparison test, * P < 0.05, *** P < 0.001, **** P < 0.0001. **(B)** Representative images of in vitro healing of scratched monolayers at 0, 18, 24, and 48 hours following treatment with buffer control, HB-EGF (250 ng/mL), and rCAP37 (25, 100, and 250 ng/mL) are shown for each time point. Images were taken using $\times 20$ objective.

time points studied (6, 24, and 48 hours), levels of proliferation were not significantly greater than vehicle-treated wounds.

CAP37 Accelerates Corneal Wound Healing in an In Vivo Mouse Model

Using the in vivo mouse model of corneal wound healing described in the Materials and Methods section, we explored the effect of topical application of exogenous CAP37 on wound closure and compared the rate of closure at 16, 24, and 48 hours with the HB-EGF positive control and the negative vehicle control (Fig. 2A). By 16 hours the wounds had effectively healed to 61% closure in the saline group and progressed to 74% closure at 24 hours. Although not studied here, it is highly probable that endogenous CAP37 and/or other related neutrophil-released proteins have a facilitating role in the wound healing process of the controls. At both time points, the HB-EGF and CAP37-treated wounds were significantly more closed than the vehicle-treated controls. To

quantify the effect of treatments on wound healing, the percents of wound closure over vehicle are shown in Figure 2B. The HB-EGF treatment significantly increases wound healing by 19 and 18% at 16 and 24 hours, respectively (P < 0.01). The CAP37 treatment significantly increases corneal wound healing by 10% and 9% at 16 and 24 hours, respectively (P < 0.05). Representative images of fluorescein-stained wounds are shown in Figure 2C and demonstrate the time-dependent healing of the wounds in response to treatment with the vehicle, HB-EGF, and CAP37. All wounds as measured by fluorescein staining indicated complete closure by 48 hours (Fig. 2C).

CAP37 Leads to Corneal Re-epithelialization

The fluorescein staining method provides a gross morphologic approach to determine the extent of corneal abrasion and healing. However, to determine whether CAP37 promotes complete re-epithelialization and restores the structural integ-

FIGURE 2. The CAP37 promotes corneal epithelial wound healing in vivo. The epithelium of the mouse cornea was removed using the Algerbrush II and corneal wounds were treated at 0 and 16 hours with HB-EGF (250 ng/mL), rCAP37 (250 ng/mL), or vehicle control (saline). Wound closure was monitored at 0, 16, 24, and 48 hours using fluorescein staining and a camera-equipped inverted microscope. Data are represented as the percent of wound closure (A) and as the percent of wound closure over vehicle (B), and are expressed as mean $\pm$ SEM. The data are representative of at least 6 mice per group. The percentage of wound closure in response to treatment was compared to vehicle-treated controls by unpaired *t*-test, $*P < 0.05$, $**P < 0.01$. (C) Representative images of mouse corneal wounds are shown at 0, 16, 24, and 48 hours following treatment with vehicle, HB-EGF, and CAP37. Dashed lines indicate the wound edge.

urity of the epithelial layers of the cornea, whole eye globes were collected at 16, 24, and 48 hours, and processed for histology (Figs. 3A–D). Hematoxylin and eosin (H&E)-stained sections revealed that re-epithelialization was well underway by 24 hours with restoration of the basal cell layers of the epithelium in response to treatment with CAP37 (Fig. 3C). The re-epithelialization in response to CAP37 (Fig. 3C) was greatly accelerated in comparison to the vehicle-treated wounds at 24 hours (Fig. 3A). The epithelium was only a single-layer thick in the central region of the vehicle-treated wound in comparison with the CAP37-treated wounds at 24 hours (Figs. 3A, 3C). However, the differentiation into squamous cells at 24 hours in response to CAP37 was not complete. This lack of complete integrity of the epithelium possibly accounted for the detection of the low level of fluorescein staining at 24 hours shown in Figure 2C. By 48 hours, the CAP37-treated wounds (Fig. 3D) had regained full integrity and could not be

histologically differentiated from the unwounded corneas (Fig. 3E). The complete re-epithelialization was reflected further in the data in Figure 2C in which no fluorescein staining was observed. The vehicle-treated cornea also had re-epithelialized by 48 hours (Fig. 3B).

PKC δ and PKC θ Are Present in Wounded HCEC Monolayers

Previous studies from our laboratory showed that PKC isoforms α , δ , ϵ , θ , η , ι , λ , and ζ are expressed in human corneal epithelial cells, and that CAP37 activates PKC δ and θ during chemotaxis.¹⁵ Since migration of epithelial cells is an important step in normal wound healing we questioned whether these isoforms were involved in wound healing. Unscratched and scratched HCEC monolayers were stained for the expression of PKC δ and θ , at 2 hours after wounding.

FIGURE 3. Histologic analysis of corneal wound closure and re-epithelialization in response to CAP37. Corneas of mice were wounded using the Algerbrush II, and treated at 0 and 16 hours with rCAP37 (250 ng/mL) or vehicle (saline). Whole eye globes were enucleated at 0, 24, and 48 hours after wounding, and sections were stained using H&E (A–E). Representative images are shown of vehicle-treated wound at 24 and 48 hours (A, B) and CAP37-treated wound at 24 and 48 hours (C, D). The extent of closure and re-epithelialization of wounds is compared with normal unwounded cornea (E). Scale bars: 100 μ m.

Results showed the constitutive expression of PKC isoforms δ and θ in unscratched HCEC monolayers and an increased staining of both isoforms along the wound edge (Fig. 4A). The constitutive expression of PKC isoforms δ and θ was confirmed using primary HCECs (Fig. 4B). The specificity of the staining for these two isoforms in the immortalized and primary HCECs was demonstrated using an IgG antibody control, which showed no staining.

CAP37 Treatment Leads to an Increase in PKC δ Staining in Wounded and Unwounded HCEC Monolayers

Our previous studies showed robust induction of PKC δ expression and translocation from cytoplasm to membrane in response to CAP37.¹⁵ By contrast, the PKC θ response to CAP37 was more ambiguous.¹⁵ We selected PKC δ for further investigation of CAP37-mediated corneal wound healing (Fig. 5). An increase in PKC δ staining was seen at 15 minutes in CAP37-treated cells in nonscratched (Figs. 5E, 5G) and scratched (Figs. 5F, 5H) monolayers over untreated non-scratched (Fig. 5A) and scratched (Fig. 5B) monolayers. Unscratched monolayers showed stronger staining with 500 ng/mL of CAP37 (Fig. 5G) than with 250 ng/mL CAP37 (Fig. 5E). There was no apparent difference in staining intensity in scratched monolayers with either dose of CAP37 (Figs. 5F, 5H). The increase in PKC δ staining in response to CAP37 was sustained, with strong staining along the wound edge observed for at least 18 hours (Fig. 5L) after treatment. Staining for PKC δ in the untreated control was minimal at this time point (Fig. 5K). The expression of PKC δ was comparable between CAP37-treated (Figs. 5E–H) and PMA-treated cells (Figs. 5C, 5D). To establish that CAP37 did not increase expression of all PKC isoforms in a nonspecific manner, we also stained for PKC α . We previously showed that this isoform was not involved in CAP37-induced HCEC chemotaxis.¹⁵ We found no increase in PKC α staining either within the monolayer (Fig. 5D) or along the wound edge (Fig. 5J).

PKC δ Is Expressed Along the Wound Edge in CAP37-Treated Corneas

Since staining for PKC δ was increased in response to wounding in cultured HCEC monolayers (Fig. 4A), studies were performed to determine whether an increase in expression of PKC δ would be seen in corneal wounds in vivo.

FIGURE 4. PKC δ and PKC θ are expressed in wounded and nonwounded HCEC monolayers. (A) HCECs (SV40 adenovirus immortalized cell line) were grown to confluency and were left unscratched (left) or scratched with a 10 μ L pipette tip (right). Monolayers were stained for PKC isoforms δ and θ at 2 hours after wounding using mouse anti-PKC δ antibody (250 ng/mL), mouse anti-PKC θ antibody (500 ng/mL), or mouse IgG control (500 ng/mL), and goat anti-mouse secondary antibody (4 μ g/mL, Alexa Fluor 488). Green punctate staining indicates positive staining for PKC δ and PKC θ . Nuclei stain blue-purple with DAPI. Representative images are shown. Scale bars: 20 μ m. (B) Primary HCECs were stained to demonstrate constitutive expression of PKC isoforms δ and θ in unwounded monolayers. Representative images are shown. Scale bars: 20 μ m.

FIGURE 5. CAP37 treatment leads to a rapid increase in PKC δ staining in HCEC monolayers. HCEC monolayers were grown to confluency and were left unscratched or scratched with a 10 μ L pipette tip and treated for 15 minutes with (A, B) buffer, (C, D) PMA (1 μ M), (E, F) 250 ng/mL CAP37 and (G, H) 500 ng/mL CAP37. Cells were stained for PKC δ (mouse anti-PKC δ at 250 ng/mL [A–H]), and PKC α (mouse anti-PKC α at 1 μ g/mL, [I, J]) and detected using immunofluorescence (goat anti mouse Alexa Fluor 488 at 4 μ g/mL). Scratched monolayers also were treated for an extended period of time (18 hours) with CAP37 (250 ng/mL, [L]) or left untreated (K). Strong staining for PKC δ was observed along the wound edge in response to CAP37 treatment (L) and was greater than that observed in the untreated wounds (K). Scale bars: 20 μ m.

Corneas were abraded as described previously and the whole mouse eye globes were collected at 6, 16, and 48 hours after wounding for immunohistochemistry to determine the expression of PKC δ in response to wounding. Little to no detection of PKC δ was observed at the leading edge of the newly proliferating and migrating epithelial cells at 6 (Fig. 6A) and 16 (Fig. 6B) hours after wounding. However, the epithelial cells at a distance from the leading edge showed a low level of staining that was comparable to the constitutive expression of PKC δ in normal unwounded cornea (Fig. 6E).

To determine if CAP37 treatment (250 ng/mL) of the wounds would have an effect on PKC δ expression, corneas were wounded and treated at 0 and 16 hours following wounding. Eyes that were enucleated at 6 and 16 hours received one treatment of CAP37 (at 0 hours), whereas the eyes that were enucleated at 48 hours received two treatments of CAP37 (at 0 and 16 hours). Strong staining for PKC δ in the newly migrating and proliferating epithelial cells as well as those cells distant from the wound edge was seen in sections taken at 6 hours after CAP37 treatment (Fig. 6C). A similar staining pattern for PKC δ was seen in sections that were obtained at 16 hours after CAP37 treatment (Fig. 6D). Sections

that were obtained at 48 hours were completely healed and showed uniform staining throughout the epithelium (Fig. 6F) and was at an intensity similar to constitutive expression of PKC δ in unwounded corneas (Fig. 6E).

PKC δ Mediates CAP37-Induced Wound Healing In Vivo

In vivo experiments were performed using siRNA to determine if CAP37 mediates wound healing via PKC δ . Mouse corneas were transfected with siRNA directed against PKC δ or with scrambled siRNA. At the end of each experiment, corneas were collected and PKC δ was quantified by Western blot. Corneas showed an average PKC δ knockdown of 50% (Fig. 7A), which was statistically significant ($***P < 0.001$) when compared to the scrambled siRNA controls.

Corneas transfected with scrambled siRNA showed a significant increase of 10% in wound closure following CAP37 treatment at 24 hours ($*P = 0.021$, Fig. 7B). In corneas transfected with PKC δ siRNA, there was no significant increase in wound healing in response to CAP37 (Fig. 7B) at either 16 ($P = 0.229$) or 24 ($P = 0.144$) hours when compared to vehicle treatment. Finally, the knockdown of PKC δ significantly decreased the effect of CAP37 on corneal wound healing measured at 24 hours ($P = 0.031$, Fig. 7B). Representative images of wounds stained with fluorescein depict the extent of wound closure over time in corneas transfected with scrambled siRNA and PKC δ siRNA and treated with CAP37 or saline (Fig. 7C). Maximum wound closure was observed in mice transfected with scrambled siRNA and treated with CAP37.

DISCUSSION

This study demonstrates a novel function for the neutrophil-derived protein CAP37, also known as heparin binding protein and azurocidin.^{18,19} Using in vitro and in vivo models of wound healing we show that CAP37 accelerates wound closure in vitro as well as in a mouse model of corneal abrasion (Figs. 1–3). By employing immunohistochemical and siRNA techniques, we established that CAP37-induced corneal epithelial wound healing occurs through the activation of the protein kinase C signaling pathway, specifically the PKC δ isoform (Figs. 4–7). To our knowledge, this is the first publication that identifies the intracellular signaling mechanism used by a neutrophil granule-derived antimicrobial protein in corneal epithelial wound healing.

The cornea is considered to be an immune privileged site²⁰ and, therefore, the process of healing in the cornea is not identical to the process that occurs in dermal wounds. However, one key feature in corneal and dermal wound healing is that neutrophils are an essential cellular component. Neutrophils are early participants in the process and are fundamental to protecting the host from infection due to their potent antimicrobial and phagocytic activity.^{21,22} When the cornea is injured, neutrophils migrate through the limbal vessels into the cornea.²³ Studies have shown that delayed corneal wound healing occurs in mice with antibody-induced neutropenia.²⁴ Other studies using wild type and knockout mice for lumican²⁵ and heme oxygenase,²⁶ and rabbit models of corneal epithelial wound healing have further established that the presence of neutrophils accelerates healing. This leads to the concept that antimicrobial proteins found within the granules of neutrophils, such as CAP37, LL-37, human β -defensin-1 (HBD-1), and bactericidal-permeability-increasing (BPI) protein may prove to be useful in modulating wound closure.

FIGURE 6. PKC δ is expressed along the leading edge of corneal epithelial wounds in CAP37-treated corneas. Corneas were wounded using the Algerbrush II, and treated at 0 and 16 hours with vehicle (saline, [A, B]) or rCAP37 (250 ng/mL, [C, D]). Whole eye globes were enucleated at 6 hours (A, C), 16 hours (B, D), and 48 hours (F) after wounding. Unwounded corneas were used as control (E). All sections were stained for PKC δ according to the details in the methods section. Representative images show constitutive staining for PKC δ in unwounded corneas (E). Wounds that were treated with vehicle show an absence of staining at the leading edge of the wound ($\downarrow$) at both time points analyzed (6 hours [A] and 16 hours [B]). Epithelial cells at a distance from the leading edge display staining for PKC δ at a similar intensity to constitutive PKC δ expressed in unwounded corneas (E). In contrast, pronounced staining for PKC δ was seen at the leading edge of the wound ($\downarrow$) in CAP37-treated corneas at 6 (C) and 16 (D) hours. PKC δ is evident in the cornea 48 hours after wounding and after re-epithelialization is complete (F). The level of staining is comparable to the staining seen at 0 hours in the unwounded cornea (E). Scale bars: 100 μ m.

Neutrophils that are recruited to the wound site release their granule contents, including CAP37 and other antimicrobial proteins and peptides that provide the first line of defense against corneal infection. It now is known that some of these antimicrobial peptides, in addition to killing the invading pathogens, are able to modulate functions of host cells that regulate innate immunity.²⁷ The neutrophil, however, is not the only source of these antimicrobial proteins. The CAP37 and LL-37 can be induced in host cells including the corneal epithelium in response to infection and wounding.^{13,24,28,29} The LL-37 has been shown, like CAP37, to be antimicrobial, bind lipopolysaccharide, and promote corneal epithelial wound healing in vitro.³⁰ Unlike CAP37, LL-37 is not known to promote HCEC proliferation³⁰ and the intracellular signaling mechanisms of LL-37-mediated corneal epithelial cell migration and wound healing have not been elucidated.

The HB-EGF was selected as a positive control because, like CAP37, it is a heparin binding protein and because there is in vitro evidence that HB-EGF facilitates corneal wound healing in organ tissue cultures.³¹ Our investigations confirmed that HB-EGF promotes wound healing in vitro and in vivo. Previous in vitro studies have indicated that HB-EGF facilitates corneal wound healing through the prolonged activation of the EGF receptor.³¹ This is believed to be due to the fact that HB-EGF is able to bind to the negatively charged glycans on the corneal surface, while EGF is washed away after treatment.³² We speculated that the heparin binding characteristics of HB-EGF that make it desirable as a long-acting alternative to EGF³¹ also may apply to CAP37, making it an effective therapeutic for ocular wound healing.

The present studies indicated that the CAP37-mediated cellular processes facilitate corneal epithelial wound healing. Time-lapse videos (Supplementary Video S3) of CAP37-treated in vitro wounds lead us to infer that CAP37 affects corneal epithelial wound healing by primarily facilitating migration, at least at the early stages of wound repair. This is supported further by the classic bell-shaped curve observed in the CAP37 dose-response experiment on wounded HCEC monolayers (Fig. 1), suggesting that CAP37-induced migration may be the

primary process leading to wound closure. Other mechanisms, such as proliferation and adhesion, may be involved at later stages of the process or when higher amounts of CAP37 are available or present in the wound milieu. These conclusions are drawn from our previous in vitro data in which we demonstrated maximal proliferation of HCEC at 72 hours with CAP37 concentrations of 1 and 2 μ g/mL.¹⁰

A significant component of our study was the delineation of the intracellular signaling mechanism that evoked CAP37-mediated corneal epithelial wound healing. We previously demonstrated that CAP37 mediates HCEC migration through the activation of a G protein-coupled receptor and the PKC isoform δ .¹⁵ In this study, the presence of PKC δ was identified in untreated corneal epithelial cell culture monolayers and confirmed in primary corneal epithelial cells (Fig. 4). The CAP37-treated corneal epithelial cell monolayers showed an increase in staining for PKC δ as early as 15 minutes (Fig. 5). We have shown previously a rapid induction of PKC δ in these cells, as early as 5 minutes after treatment with CAP37.¹⁵ Others have shown a surprisingly fast increase of PKC δ transcription and translation in muscle cells within a few minutes of insulin treatment.³³ In our experiments the induction of PKC δ was not transient and persisted up to 18 hours after CAP37 treatment (Fig. 5). In vivo studies using immunohistochemistry, show strong expression of PKC δ along the leading edge of the wound in response to CAP37 treatment (Fig. 6). The presence of PKC δ along the wound edge and an increase in PKC δ in CAP37-treated wounds, prompted studies in which PKC δ was knocked down in a mouse model of corneal epithelial wound healing. Results revealed that a partial knockdown of PKC δ is sufficient to significantly reduce the effect of CAP37 on corneal wound healing in vivo, although not completely ablating it (Fig. 7). The average knockdown of PKC δ was 50% and could explain why CAP37 still promoted a certain amount of wound closure in corneas transfected with siRNA directed against PKC δ . Another explanation for these findings is that other PKC isoforms such as PKC θ may partially contribute to CAP37-mediated wound healing.

FIGURE 7. PKC δ mediates CAP37-induced wound healing in vivo. **(A)** siRNA directed against PKC δ or scrambled siRNA was injected into the mouse conjunctiva before corneal abrasion. The efficiency of the PKC δ knockdown in each cornea was confirmed by Western blot analysis at 24 hours after injection and was compared to levels observed in corneas injected with the scrambled siRNA after normalization to β actin. Data are representative of 22 experimental samples and are expressed as mean $\pm$ SEM (*** P < 0.001 by unpaired t -test). **(B)** Corneal abrasions were treated at 0 and 16 hours with rCAP37 (250 ng/mL), or were left untreated (saline control). Wound healing was monitored at 0, 16, and 24 hours using fluorescein staining. Data are represented as the percent of wound closure over vehicle and are expressed as mean $\pm$ SEM. The data are representative of at least 9 mice per group. Unpaired t -test was used to compare CAP37 to vehicle (saline)-treated controls, and to compare scrambled and PKC δ siRNA groups after treatment with CAP37. **(C)** Representative images stained with fluorescein indicating extent of closure of corneal abrasions in response to vehicle and CAP37-treatment at 0, 16, and 24 hours in mice receiving conjunctival injection of scrambled siRNA and PKC δ siRNA. The dotted line is used to demarcate the edge of the wound.

Corneal epithelial wound healing is a complex process involving many cellular players, growth factors, chemokines, and mediators. Due to its cellular localization, effects on corneal epithelial cells, and recruitment of inflammatory cells, we believe that CAP37 is an important mediator of corneal epithelial wound healing. A signaling mechanism involved in CAP37-mediated wound healing has been identified in these

studies. The next step in the understanding of the role of CAP37 in corneal wound healing is to define the cytokines, chemokines, and inflammatory cells that are regulated by CAP37. Delineation at the cellular and molecular level of the contribution of CAP37 to this complex and multistep process will help develop potential new therapeutics for corneal wound healing and infections.

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