Multifunctional Roles of Human Cathelicidin (LL-37) at the Ocular Surface

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PURPOSE. The goals of this study were to examine the expression of the antimicrobial peptide LL-37 in the corneal epithelium during wound healing and to investigate whether LL-37 stimulates human corneal epithelial cell (HCEC) migration, proliferation, and cytokine production.

METHODS. Expression of LL-37 was determined by RT-PCR and immunostaining in tissue sections and HCECs scraped from corneas before (original) and after (regrown) re-epithelialization. The antimicrobial activity of LL-37 against Pseudomonas aeruginosa (PA) was determined in the presence of NaCl and tears. Blind-well chamber assays were performed to study the effect of LL-37 on migration. Proliferation was determined using calcein-AM, and cytotoxicity was evaluated by MTT assay. ELISA was performed to assess the ability of LL-37 to stimulate HCEC cytokine secretion.

RESULTS. LL-37 peptide was present throughout the corneal epithelium (n = 4). All original corneal epithelial samples expressed a low level of LL-37 (n = 10). Regrown epithelial samples collected 24 (n = 3 of 5) or 48 (n = 4 of 5) hours after wounding showed upregulated expression of LL-37. LL-37 killed PA in the presence of NaCl and tears. Blind-well chamber assays were performed to study the effect of LL-37 on migration. Proliferation was determined using calcein-AM, and cytotoxicity was evaluated by MTT assay. ELISA was performed to assess the ability of LL-37 to stimulate HCEC cytokine secretion.

CONCLUSIONS. LL-37 expression is upregulated in regenerating human corneal epithelium, has antibacterial activity against ocular pathogens under physiologically relevant conditions, and stimulates HCEC migration and cytokine production. These findings suggest that LL-37 acts as a multifunctional mediator that helps protect the cornea from infection and modulates wound healing. (Invest Ophthalmol Vis Sci. 2006; 47:2369–2380) DOI:10.1167/iovs.05-1649

The ocular surface is protected against microbial invasion by both the innate and adaptive immune systems. In addition to corneal and conjunctival epithelia that form a protective barrier, antimicrobial enzymes, and other proteins in the tear film are also essential components of the innate defense response. Recent studies have shown that epithelial cells in various tissues, including those of the cornea and conjunctiva, secrete antimicrobial peptides such as β-defensins and cathelicidins, which are presumed to help protect the eye against a wide range of microorganisms.

The cathelicidins comprise a highly conserved region (the cathelin domain) and a less-conserved antimicrobial region that varies among species, yielding multiple peptides with a remarkable variety of sizes, sequences, and structures. LL-37, a unique antimicrobial peptide consisting of 37 amino acids, is derived from human cationic antimicrobial protein 18 (hCAP18) and is the only cathelicidin described in humans. Initially found in bone marrow, LL-37 was later isolated from immune cells including neutrophils and lymphocytes. LL-37 is also expressed by epithelial cells in the oral cavity and the respiratory, urogenital, and gastrointestinal tracts, findings that are in keeping with the protective role of epithelial tissue. LL-37 is capable of stimulating IL-8 (a neutrophil chemoattractant) secretion by lung and skin epithelial cells. To date, several receptors associated with LL-37-mediated immunomodulation, including N-formylpeptide receptor-like-1 (FPRL1), purinergic receptor P2X₇, and epidermal growth factor receptor (EGFR), have been identified. Although growing evidence suggests that LL-37 acts in a receptor-dependent fashion, the exact mechanisms of how it exerts its nonantimicrobial functions are yet to be determined.

A recent study demonstrated that LL-37 expression is induced in skin wounds and that the peptide may stimulate cell proliferation to enhance wound re-epithelialization. Furthermore, LL-37 has been implicated as an angiogenic factor, stimulating vascular endothelial cell proliferation and thereby promoting wound healing. In addition, Shaykhiev et al. have reported that LL-37 induces wound healing of airway epithelial cells by stimulating cell migration and proliferation. These new lines of evidence suggest that LL-37 may be involved in modulating cell behavior essential for wound repair. Previously, we studied LL-37 expression in the ocular surface epithelia and observed that cultured human corneal and conjunctival epithe-
lial cells and freshly scraped corneal epithelium express a low level of LL-37, an observation recently confirmed by McIntosh et al., and that this expression is upregulated in conditions mimicking inflammation (Huang LC, et al. IOVS 2003;44: ARVO EAbstract 1335). Given that there is strong evidence of a role for LL-37 in wound healing, we hypothesize that this peptide, in addition to being antimicrobial, may be involved in the process of epithelial regeneration at the ocular surface.

To investigate further the potential functions of LL-37 at the ocular surface, we studied the expression of LL-37 in regenerating corneal epithelium during wound healing and the ability of the peptide to modulate human corneal epithelial cell (HCEC) migration, proliferation, and cytokine secretion, all of which are essential in epithelial wound repair. We also examined the antimicrobial activity of LL-37 under physiologically relevant salt concentrations and in the presence of human tears. Preliminary findings in this study have been presented in abstract form (Huang LC, et al. IOVS 2004;45:ARVO EAbstract 4940).

**Materials and Methods**

**Immunostaining for LL-37**

Human corneas were obtained from Lions Eye Banks (Central Florida and Heartlands) and used in accordance with the tenets of the Declaration of Helsinki regarding the use of human tissue in research. The maximum elapsed time between donor death and receipt of the tissue was 5 days. The mean age of the donors was 68 ± 2 years. The corneas were embedded in optimal cutting temperature (OCT) compound, frozen, and sectioned (15 µm) on a cryostat. The sections were fixed in 4% paraformaldehyde and incubated with blocking solution (0.1% goat serum, 0.05% gelatin, and 0.05% Tween-20 diluted in PBS). After blocking, the sections were incubated with a rabbit anti-LL-37 polyclonal antibody diluted 1:500 (a gift of Robert Lehrer, University of California Los Angeles) at 4°C overnight and then with a cy3-conjugated second antibody diluted 1:300 in blocking solution. Sections from which the anti-LL-37 antibody was omitted served as the background control. The slides were viewed under a microscope equipped for digital fluorescence imaging.

**In Vitro Corneal Epithelial Wounding**

The epithelial wounding procedure was adapted from Foreman et al. The tissue was mounted on a silicon mold, and the epithelium was scraped off (“original” epithelial sample) with a scalpel blade, leaving an intact 1- to 2-mm band around the limbus. The endothelial cavity of each cornea was filled with M199 containing 0.5% agar and then displaced epithelial side up in organ culture in a 35-mm culture dish filled with M199 to the level of the limbal conjunctiva. The corneas were maintained at 37°C, and their epithelial surfaces were kept moist by drop-wise (approximately 50 µL) every 8 hours. At 24 or 48 hours, the tissue was scraped off (“regrown” epithelial sample). The antimicrobial peptide, LL-37, was purchased from American Peptide (Sunnyvale, CA), and 50 µg/mL gentamicin. 

**Reverse Transcription–Polymerase Chain Reaction**

Total RNA from cell samples was extracted with an RNaseq kit (Qiagen). Total RNA (250 ng) was used in each RT-PCR reaction (SuperScript II; Invitrogen, Carlsbad, CA). Reactions containing normal human testis RNA (BD-Clontech Laboratories, Palo Alto, CA) or RNase-free water in place of the RNA are used as positive and negative controls, respectively. In some reactions, the reverse transcriptase was eliminated (∼RT control). Reverse transcription was performed at 50°C for 60 minutes. After denaturation of the enzyme (94°C, 5 minutes), amplification of the cDNA was performed for 40 cycles: denaturation, 94°C for 50 seconds; annealing, 60°C (FPRL1) or 62°C (LL-37) for 30 seconds; and extension, 72°C for 1 minute. The specific primers used were β-actin26: forward 5′-GGATCTTCATGAGGTAGTCAGTC-3′ and reverse 5′-GGAATTCGGATCCTGAGTC-3′; and FPRL1 25: forward 5′-ATCTGTCTCAATGGGAGGTAGTCAGTC-3′ and reverse 5′-GTCCTGCCATACCAGCTCTTC-3′. Products generated with these primers were sequenced (Seqwright, Houston, TX) to confirm their identities. A commercial base pair marker (HyperLadder I; Midwest Scientific, St. Louis, MO) was used.

**Immunoblotting Analysis**

Immunoblots were performed to detect LL-37 peptide according to the procedure described previously. Epithelial samples were homogenized in 100 µL of ice cold Tris-buffered saline (TBS: 150 mM NaCl, 20 mM Tris-HCl [pH 7.5]). Cell lysate (25 µg of total protein) was blotted onto a nitrocellulose membrane using a microfiltration apparatus (Bio-Dot, Irvine, CA). Five nanograms of synthetic LL-37 peptide were also blotted onto the membrane as a positive control. The membranes were blocked in blocking solution (5% milk, 0.9% NaI in PB), incubated with a rabbit anti-LL-37 polyclonal antibody diluted 1:5,000 in blocking solution (3% milk, 0.9% NaI in PB) overnight, and then incubated with a horseradish peroxidase–linked second antibody diluted 1:10,000 in blocking solution. Immunoreactivity was visualized by enhanced chemiluminescence (ECL Plus Western Blot Detection kit; GE Healthcare, Piscataway, NJ).

**LL-37 Peptides**

The antimicrobial peptide, LL-37, was purchased from American Peptide Company (Sunnyvale, CA) and used in all the experiments. A scrambled peptide of LL-37 with the same amino acids arranged randomly was obtained from Global Peptide Services (Fort Collins, CO). Synthetic and scrambled LL-37 peptide were dissolved in 0.01% acetic acid at a concentration of 1 mg/mL and stored at −20°C.

**Preparation of Pseudomonas aeruginosa**

_Pseudomonas aeruginosa_ (PA; 27853; ATCC; Manassas, VA) was tested in this study. This ATCC strain is known to invade the cornea and produce severe ocular infection in experimentally infected animal models of bacterial keratitis. One single isolated PA colony was used to inoculate 5 mL of nutrient broth (NB) overnight at 37°C. Fifty microliters of this bacterial suspension were used to inoculate 50 mL of...
fresh NB, which was then incubated for 2.5 hours with vigorous shaking at 37°C to achieve mid-log phase growth. Twenty-five milliliters of the warm PA culture were centrifuged at 3100g for 10 minutes, and the bacterial cell pellet was resuspended in cold phosphate buffer (PB; 8.2 mM Na3HPO4, 1.8 mM KH2PO4; pH 7.4). Optical density of the suspension was adjusted to 0.2 at 620 nm (approximately 10^7 cfu/mL) by adding an appropriate volume of PB.

**Tear Sample Collection from Human Subjects**

All procedures involving human subjects were performed with the approval of the University of Houston Institutional Review Board and in accordance with the tenets of the Declaration of Helsinki regarding research involving human subjects. All subjects had a complete ophthalmic examination at the University Eye Institute (University of Houston) and were found to be free of any ocular surface disease. Three subjects (2 men, 1 woman; age range, 26–35 years) took part in the study. Informed consent was obtained from all subjects after explanation of the nature and possible consequences of the study. Unstimulated tears were collected from the inferior tear meniscus into 5-μL microcapillary tubes (Drummond Scientific, Broomall, PA). Anesthetizing the ocular surface can lead to a reduction in tear production, therefore, tears were collected without the use of anesthetics.48 Eighty to 100 μL of tears were collected from each subject over a total of three visits spaced 2 to 3 days apart. Tear samples were stored at –80°C until use.

**Antimicrobial Activity of LL-37**

The antimicrobial assay procedure was adapted from that described by Tomita et al.37 Reaction mixtures (final volume 50 μL) containing 10 μL of 10^-1 cfu/mL PA and 5 μL LL-37 (final concentration 0.5, 1, 0.1, 0.5, 1, 10, 25, 50, and 100 μg/mL) diluted in PB or 150 mM NaCl solution were incubated at 37°C for 2 hours with vigorous shaking. In each experiment, reaction mixtures containing 5 μL of 0.01% acetic acid, the vehicle for diluting LL-37, acted as a control. In addition, in each experiment, the susceptibility of the organism to ciprofloxacin ophthalmic solution (5 μL of 0.3% solution; Ciloxan; Alcon, Fort Worth, TX), a topical agent commonly prescribed to treat ocular PA infection, was tested. At the end of the incubation, serial dilutions of each reaction mixture were used to inoculate NB agar plates. Samples (10 μL) were spread evenly over the surface of the plates with sterile glass spreaders. After incubation at 37°C for 24 hours, the agar plates were placed on a light board and a digital image captured (Alpha Imager documentation system; Alpha Innotech). The number of colonies was counted using the colony count software of the system. The ECO50 (the effective concentration that resulted in 50% killing of PA) was calculated (GraphPad Prism® software; GraphPad Software, San Diego, CA).

Additional experiments were also performed to test the antimicrobial activity of LL-37 in the presence of human tears. Antimicrobial assays were performed with 3 μg/mL LL-37 (previously determined ECO50 in the absence of high salt) and 100 μg/mL LL-37, which was 100% effective in killing PA. Tears were diluted in PB to give final reaction mixtures containing 70% vol/vol tears. Because of other constituents of the reaction mixture, 70% vol/vol was the maximum tear concentration obtainable in these experiments.

**Blind-Well Chamber Migration Assay**

Assays were performed using polycarbonate membranes of 10- to 12-μm pore size (Neuroprobe, Gaithersburg, MD). LL-37 and the scrambled peptide (final concentration range, 0.1–20 μg/mL) were diluted in serum-free culture medium and placed in the bottom chambers of the blind wells. P-HCECs or SV-40-transformed HCECs in serum-free culture medium were placed into the top chambers (10^4 cells/chamber). In some experiments HCECs were pretreated with pertussis toxin (PTX, 100 and 250 ng/mL) for 2 hours, or with Wrw4 (Trp-Arg-Trp-Trp-Trp-CONH2 or WRRWWW, an FPRL1 peptide antagonist, 10 or 50 μM) or inhibitors of various cellular signaling pathways: PD98059 (an ERK1/2 inhibitor, 20 μM), SB203580 (a p38 MAPK inhibitor, 5 μM), SP600125 (a c-JNK inhibitor, 25 μM), H-7 (a PKC inhibitor, 100 μM), LY294002 (a PI3K inhibitor, 50 μM), genistein (a TK inhibitor, 50 μM), and AG1478 (an EGFR-TK inhibitor, 0.1, 1, and 10 μM) for 15 minutes. All inhibitors were purchased from Calbiochem with the exception of SP600125 and H-7 which were obtained from Sigma-Aldrich. Concentrations of various inhibitors were selected based on effective ranges established by previous studies using other cell types. Culture media containing fibronectin (2 μg/mL) added to the bottom chamber acted as a positive control. The chambers were incubated at 37°C for 16 hours, and the membranes were removed, fixed in methanol, and stained with HEMA 3 solutions I and II. The number of migrated cells in five randomly selected high-power fields of each membrane was counted by light microscopy. The data were analyzed by Student’s t-test with P ≤ 0.05 being considered significant.

**Proliferation Assay**

P-HCECs (1500/cell in well in 96-well plates) were allowed to grow for 48 hours and then starved in the absence of HCGS for 48 hours, to arrest proliferation. Cells were exposed to HCGS-free media containing LL-37 (0.001, 0.01, 0.05, 0.1, 0.5, 1, 2.5, or 5 μg/mL). Untreated control cells were exposed only to HCGS-free growth medium, whereas positive control cells received medium containing HCGS. Six replicates were prepared for each of the conditions. LL-37 was replenished every 24 hours. After incubation (48 hours, 37°C), cell proliferation was assessed with the fluorescent dye calcein-AM (Invitrogen, Eugene, OR). Cells were incubated with calcein-AM (5 μg/mL) for 1 hour, and the fluorescence was measured on a plate reader (HTS7000; PerkinElmer, Shelton, CT). The data collected were analyzed by one-way ANOVA (post hoc Scheffé comparison) with P ≤ 0.05 considered significant.

**MTT Cytotoxicity Assay**

P-HCECs or SV40-HCECs were grown to confluence in 96-well plates. LL-37 and the scrambled peptide (final concentration range 1–50 μg/mL) were diluted in serum-free culture medium and then incubated with HCECs for 24 hours. Control cells were exposed to either culture medium alone for 24 hours or to 0.002% benzalkonium chloride for 30 minutes (positive control). Five replicates were prepared for each of the conditions. Cytotoxicity was assessed with an MTT assay kit (Chemicon International, Temecula, CA), according to the manufacturer’s instructions. The data were analyzed by Student’s t-test with P ≤ 0.05 considered significant.

**ELISA for IL-8, IL-6, IL-1β, and TNF-α**

P-HCECs were cultured in six-well plates to near confluence and then incubated with LL-37 (final concentration 0.0001, 0.001, 0.01, 0.1, 0.5, 1, and 5 μg/mL) diluted in HCGS-free culture medium or with medium alone as the control for 6 and 24 hours. In some experiments, cells were pretreated with signaling pathway inhibitors for 15 minutes at concentrations shown to be effective in inhibiting LL-37 stimulated HEC migration: Wrw4 (50 μM), PD98059 (10 μM), SP600125 (10 μM), LY294002 (50 μM), genistein (50 μM), and AG1478 (10 μM). Cell-free supernatants and cell pellets collected at each time point were immediately stored at –80°C for ELISAs and Bradford assays, respectively. IL-1β, IL-6, IL-8, and TNF-α levels in supernatant samples were measured in triplicate for each sample with specific ELISA kits (R&D System, Minneapolis, MN), according to the manufacturer’s instructions. The data were analyzed by Student’s t-test with, P ≤ 0.05 considered significant.

**RESULTS**

LL-37 and the Ocular Surface

LL-37 and hCAP18 mRNA and Peptide Expression in Normal and Regenerating Corneal Epithelium

Immunostaining was performed to localize LL-37 peptide in normal human corneal sections. LL-37 immunoreactivity was detectable throughout the corneal epithelial layer as shown in
representative images (Fig. 1A) from one of four corneas. Immunoreactivity was not present in background control samples in the absence of the primary antibody. RT-PCR was performed to study LL-37/hCAP18 mRNA expression in original and regrown human corneal epithelium (Fig. 1B). All original epithelial samples \((n = 10)\) expressed a low level of LL-37/hCAP18 mRNA. Three of five regrown epithelial samples collected 24 hours and four of five collected 48 hours after wounding showed that LL-37/hCAP18 mRNA expression was upregulated approximately 2.4-fold \((P < 0.05)\) compared with the original samples. LL-37/hCAP18 to β-actin signal ratios were 0.09 ± 0.03 and 0.24 ± 0.09 (24 hours) and 0.11 ± 0.02 and 0.27 ± 0.03 (48 hours) in original and regrown samples, respectively. Immunoblot analysis was performed to study LL-37 peptide expression in original and regrown human corneal epithelial samples collected 48 hours \((n = 4)\) after wounding. The results from two representative pairs are shown in Figure 1C, which shows that LL-37 peptide expression increased in regenerating corneal epithelium. Comparable results were seen with two other corneal pairs.

**Effect of NaCl and Human Tears on the LL-37 Activity against PA**

We have previously studied the antimicrobial activity of LL-37 against PA (ATCC 27853) under standard conditions.\(^{15}\) In the current study, antibacterial assays were performed to study the activity of LL-37 against this strain under more physiological conditions. As shown in Figure 2A, LL-37 inhibited the growth of PA in a concentration-dependent manner, but when tested in the presence of 150 mM NaCl, activity of lower concentrations of LL-37 (0.05–10 \( \mu \)g/mL) was moderately impaired, with the EC\(_{50}\) for killing PA being reduced from 2.8 ± 1.5\(^{15}\) to 10.3 ± 2.5 \( \mu \)g/mL in the presence of NaCl. High concentrations of LL-37 (50–100 \( \mu \)g/mL) completely killed PA and were as effective as ciprofloxacin (data not shown), even in the presence of NaCl. When tested in the presence of human tears (Fig. 2B), activity of 3 \( \mu \)g/mL LL-37 (mean EC\(_{50}\) of LL-37 established previously\(^{15}\)) was moderately reduced similar to the extent found in the presence of NaCl. Similar to the findings observed with NaCl, the activity of a high concentration of
LL-37 (100 μg/mL) was not altered in the presence of human tear fluid.

**Effect of LL-37 on HCEC Migration, Proliferation, and Cytokine Secretion**

To determine whether LL-37 plays a role in influencing cell behavior during epithelial wound healing, we studied the effect of LL-37 on HCEC migration, proliferation, and cytokine secretion. As shown in Figure 3A, LL-37 stimulated P-HCEC migration and this effect was concentration dependent. Fibronectin, as a positive control, also stimulated P-HCEC migration, whereas culture media did not. Significant effects were observed at LL-37 concentrations equal to or above 1 μg/mL (n = 5, P ≤ 0.01, Student’s t-test). Scrambled peptide did not stimulate P-HCEC migration at any of the concentrations tested (n = 3, data not shown). Comparable data were obtained with SV40-HCECs (data not shown). Culture media or LL-37 alone did not stimulate HCEC proliferation (Fig. 3B), whereas HCGS (a positive control) did. To study the effect of LL-37 on chemokine and cytokine secretion in corneal epithelium, cell-free supernatants collected from P-HCECs treated with and without various concentrations of LL-37 (0.0001, 0.001, 0.01, 0.1, 0.5, 1, and 5 μg/mL) for 24 hours were tested for IL-8, IL-6, IL-1β, and TNF-α by chemokine/cytokine-specific ELISAs (Fig. 4).

**Effect of LL-37 on HCEC Migration**

As nonmicrobicidal effects of LL-37 have been reported to be receptor mediated, we investigated whether HCECs express FPRL1, a known receptor for LL-37.17 RT-PCR was performed to study FPRL1 expression in HCECs (Fig. 5A). Both SV40-HCECs and P-HCECs expressed FPRL1 mRNA (n = 3, each). FPRL1 is a receptor linked to a Gi protein. To determine whether LL-37-induced migration is mediated via a G-protein-coupled receptor (GPCR) such as FPRL1, we studied the effect of pertussis toxin (PTX, a Gi inhibitor). PTX alone did not affect cell migration (data not shown). As shown in Figure 5B, pretreatment with PTX either partially (100 ng/mL PTX) or almost completely (250 ng/mL PTX) eliminated LL-37-stimulated SV40-HCEC migration (n = 3, P < 0.05, Student’s t-test), suggesting involvement of a GPCR. To determine whether FPRL1 was the
inositol-3 kinase (PI3K), and epidermal growth factor receptor kinase, c-JNK; extracellular signal-regulated kinase, ERK1/2), activated protein kinase (MAPK; p38MAPK; c-Jun-N-terminal kinase, c-JNK; extracellular signal-regulated kinase, ERK1/2), and PKC. When tested alone, these inhibitors had no inhibitory effect (Fig. 4B). As shown in Figure 4B, the TK inhibitor genistein partially (by 51% inhibition) and AG1478 almost completely (up to 88% inhibition) blocked the stimulatory effect of LL-37 on cell migration by 60% and 78%, respectively. LY294002, PD98059, and SP203580 all effectively (by 73%, 74%, and 60% inhibition, respectively) attenuated LL-37 mediated IL-8, IL-6, and IL-1 \( \beta \) production in HCECs. ELISA was performed to measure IL-8 (A), IL-6 (B), IL-1 \( \beta \) (C), and TNF-\( \alpha \) (D) release in cell-free supernatant from P-HCECs treated with LL-37 (0.0001, 0.001, 0.01, 0.5, 1, or 5 \( \mu \)g/mL) for 24 hours, whereas cells treated with media served as control (C). Results are expressed as the mean (pg/mg cell protein) \( \pm \) SEM of one representative experiment repeated three to four times, each performed in triplicate. *P < 0.05 compared with the control.

Effect of Selected Signaling Pathway Inhibitors and Receptor Antagonists on LL-37-Induced HCEC Cytokine Secretion

To investigate further whether LL-37-induced HCEC chemokine and cytokine production is mediated through the multiple signaling pathways involved in stimulating cell migration, we pretreated HCECs with LY294002, PD98059, SP203580, genistein, AG1478, and WRW4 and studied the effect of these inhibitors on HCEC cytokine release in the presence and absence of LL-37. Cell-free supernatants collected from inhibitor-treated P-HCECs incubated with LL-37 (5 \( \mu \)g/mL) for 24 hours were tested for IL-8, IL-6, IL-1 \( \beta \), and TNF-\( \alpha \) by chemokine/cytokine-specific ELISA (Figs. 7, 8). HCECs receiving only media or inhibitors alone (data not shown) produced low levels of all proinflammatory cytokines tested. LY294002, PD98059, and SP203580 all effectively (by 73%, 74%, and 60% inhibition, respectively) attenuated LL-37 mediated IL-8, IL-6, IL-1 \( \beta \), and TNF-\( \alpha \) secretion (Fig. 7; n = 2–3). As shown in Figure 8, genistein also partially (by 51% inhibition), and AG1478 almost completely (up to 88% inhibition) blocked LL-37-stimulated IL-8, IL-6, IL-1 \( \beta \), and TNF-\( \alpha \) secretion, whereas WRW4 showed no inhibitory effect (n = 2–3).

Effect of High Concentrations of LL-37 on HCECs

A possible cytotoxic effect of LL-37 on P-HCECs was studied by using an MTT-based cell-survival assay (Fig. 9). Media containing benzalkonium chloride (BAC, positive control) caused significant HCEC death (P < 0.005, Student’s \( t \)-test), whereas low concentrations of LL-37 (≤10 \( \mu \)g/mL) showed no toxic effect on the HCECs. When tested at higher concentrations, LL-37 (≥10 \( \mu \)g/mL) became cytotoxic to HCECs in a concentration-dependent manner (n = 3, P < 0.05, Student’s \( t \)-test). Scram...
known LL-37 receptor. Data are results of one representative experi-
mentation with SV40-HCECs (data not shown). Identical results were obtained when the experiment was performed with SV40-HCECs (data not shown).

**DISCUSSION**

Recent studies have shown that human corneal and conjunctival epithelia express LL-37. In this study, we investigated the expression of LL-37 in normal and regenerating corneal epithelia during wound healing in vitro and addressed the potential functional roles of LL-37 in the corneal epithelium during wound repair. Our data show that human corneal epithelial cells expressed a low level of LL-37 mRNA and peptide, and this expression was upregulated during re-epithelialization. The exact mechanisms that regulate the expression of LL-37 at the ocular surface remains to be determined. It has been demonstrated in other tissues that LL-37 expression is increased in response to inflammation and infection, and this expression by various types of epithelial cells is inducible on stimulation with proinflammatory cytokines and bacterial components. Notably, we have found that LL-37 expression is increased by the inflammatory cytokine interleukin (IL)-1β (Huang LC, et al. *IOVS* 2003;44:ARVO E-Abstract 1335) and by heat-killed PA (Huang and McDermott, unpublished observation, 2003). Therefore, we expected to see a differential pattern of expression between normal and regenerating corneal epithelium, as cytokines such as IL-1β are known to be increased after corneal injury. We postulate that upregulated expression of LL-37 during corneal wound healing may serve as a protective mechanism and that LL-37 has important roles as an antimicrobial peptide and may be involved in epithelial wound healing at the ocular surface.

Previously, we have studied the antimicrobial activity of LL-37 against various common ocular pathogens including *P. Staphylococcus aureus*, and *Staphylococcus epidermidis*. By virtue of their cationic nature, antimicrobial peptides such as LL-37 disrupt the anionic microbial cell membrane through electrostatic interaction which increases permeability of the membrane causing cell death. The presence of NaCl can interrupt this process. Indeed, it has been shown that the antimicrobial activity of some cationic peptides, including that of LL-37, is attenuated in the presence of high salt content, although this is dependent on the concentration of peptide being used, with higher concentrations being little affected. Rationalizing that the salt content of human tears may interfere with the activity of LL-37, whether endogenously expressed or exogenously applied, we studied the effect of physiological salt concentration (150 mM NaCl, comparable to that in the tear fluid) on LL-37 antibacterial activity. It has been established that the MIC value of LL-37 is between 1 to 31 μg/mL against various microorganisms in medium of low ionic strength, and we calculated the EC50 for killing of PA to be approximately 2.8 μg/mL. We observed that this activity is moderately reduced (EC50 of 10.3 μg/mL) when tested in the presence of physiological NaCl concentration. It is noteworthy that when tested in the presence of human tears, the activity of a low concentration (EC50) of LL-37 is comparatively reduced to the level observed in the presence of NaCl, whereas high concentrations of LL-37 maintain a strong activity against PA.

The actual concentration of LL-37 at the ocular surface in vivo has yet to be determined, although we hypothesize that the peptide is significantly expressed during infection, during inflammation, and after injury. Studies have reported that this peptide is present at concentrations around 2 μg/mL in other epithelial cells. Schaller-Bals et al. have established the physiological
concentrations of LL-37 at a site of lung infection or inflammation to be approximately 1 μM (~5 μg/mL), a concentration that is antimicrobial and also stimulates lung epithelial cells to secrete chemokines. Presumably a concentration gradient of LL-37 may be formed at the ocular surface as a result of release by neutrophils and production by epithelial cells which can reach high levels through local sequestering effects in the tear film and extracellular fluid. However, one confounding factor we have observed is of high concentrations (>10 μg/mL) of LL-37 being cytotoxic to human corneal epithelial cells. Similarly, other studies have shown high concentrations of LL-37 to be cytotoxic to various mammalian cell types. Based on our EC50 calculations, the concentration of LL-37 that would be required for adequate killing at the ocular surface would also produce significant cytotoxicity resulting in tissue damage. However, such high concentration may not actually be required for LL-37 antimicrobial activity. To date, synergies between LL-37 and host defense proteins and other antimicrobial peptides such as defensins, lactoferrin, and lysozyme (all previously found to be present in the tear fluid) have been demonstrated in terms of antimicrobial activity. These synergistic interactions appear to help overcome some of the detrimental effects of high salt and may allow for significant antimicrobial activity at lower peptide concentrations while avoiding cytotoxic effects. In addition, it has been shown that certain cytokines present at mucosal surfaces appear to synergize with LL-37 to enhance its immunomodulatory activity. These findings suggest that lower concentrations of LL-37 are sufficient to perform its multifunctional roles as an antimicrobial and immune modulator.

LL-37 has been implicated to modulate various cell behavior and functions, in particular migration and proliferation which are essential for proper epithelial wound repair. Studies have indicated that LL-37 exerts its immunostimulatory effect on cellular behavior through receptor-mediated pathways, and, therefore, NaCl would not alter the non-antimicrobial activities of the peptide. Several studies have recently reported that LL-37 induces migration, and expression and release of numerous inflammatory mediators by various cell types including leukocytes, keratinocytes, and lung epithelial cells. Similarly, we have observed that LL-37 induced human corneal epithelial cell migration and stimulated IL-8, IL-6, IL-1β, and TNF-α production. These chemokine and cytokines may in turn regulate corneal epithelial cellular activities. In the context of wound re-epithelialization, IL-6 has been shown to stimulate epithelial cell migration, whereas IL-1β has been shown to facilitate epithelial wound closure in the cornea. In the process of combating infection, IL-8 has been demonstrated to help recruit neutrophils, monocytes, and T-lymphocytes. Both IL-1β and TNF-α are also capable of initiating a series of immunomodulatory chain reactions influencing the behavior of cells of the innate and adaptive immune systems at the ocular surface. LL-37, however, had no effect on corneal epithelial cell proliferation when tested over the concentration ranges of LL-37.

Cells treated with medium served as the control. (A) The effect of inhibitors of the three MAP kinase pathways: SB (SB203580, p38 MAPK inhibitor); PD (PD98059, ERK1/2 inhibitor); SP (SP600125, c-JNK inhibitor). (B) The effect of LY (LY294002, PI 3K inhibitor), H-7 (PKC inhibitor), and genistein (TK inhibitor). (C) The effect of the EGFR-TK inhibitor AG1478. Pretreatment of cells with all inhibitors tested had no effect on cell migration (data not shown). Data shown are expressed as mean ± SEM of five chambers per condition and are from three representative experiments. *P < 0.05 compared to LL-37 alone.

Figure 6. Effect of intracellular signaling inhibitors on LL-37-mediated HCEC migration. Blind-well migration assays were performed in the presence and absence of LL-37 (5 μg/mL), using SV40-HCECs pretreated with and without various intracellular signaling inhibitors.
range that stimulated both migration and cytokine secretion. Taken together, our observations raise the possibility that the roles of LL-37 at the ocular surface may be several, including to promote wound repair by enhancing epithelial cell migration and cytokine secretion and also through chemokine/cytokine production to modulate the innate and adaptive immune responses.

It is not entirely clear how LL-37 exerts its effects on corneal epithelial cell behavior, although studies have suggested that LL-37 effects on immune cells, keratinocytes,

airway epithelial cells, and other cell types are mediated by specific receptors linked to intracellular signaling pathways. Recent published data by Niyonsaba et al. suggested that LL-37 induced effects on mammalian cell function via an as-yet-unidentified receptor coupled to a Gi protein-phospholipase C (PLC) signaling pathway. Lau et al. have proposed a direct receptor-binding interaction of LL-37 to the epithelial cell surface and subsequent internalization via endocytosis. Other studies have recently proposed molecular mechanisms of EGFR involvement in LL-37 induced cellular activities. The means by which LL-37 activates EGFR have recently been hypothesized, and it is suggested that activation of a metalloproteinase by LL-37 leads to release of EGFR ligands (e.g., heparin binding EGF) which in turn transactivate EGFR. To date, FPR1, a pertussis toxin-sensitive GPCR, is the only receptor identified to which LL-37 may bind directly. Evidence for expression of functional FPR1 comes from murine corneas where lipoxin A4 (an FPR1 agonist) was shown to be involved in promoting epithelial wound healing and host defense. We have now discovered that human corneal epithelium expresses FPR1 mRNA and that PTX inhibits LL-37-induced migration. It has been well established that HCECs express EGFR, and transactivation of this receptor has been noted. These observations suggest that HCECs express FPR1- and EGFR-mediated pathways by which LL-37 could directly influence corneal epithelial cell behavior.

We investigated the participation of FPR1 and EGFR in LL-37 mediated HCEC migration. The FPR1-antagonist, WRW4 and AG1478 independently exerted considerable inhibitory effects on LL-37 simulated cell migration at the concentrations tested indicating contributions from both FPR1 and EGFR. Cross-communication between EGFR and GPCRs has been described previously, and, therefore, may be the explanation of the inhibitory effects of both PTX and AG1478 on corneal epithelial cell migration. Previous studies also have shown involvement of EGFR/GPCR in LL-37 stimulated migration. This peptide was demonstrated to be a chemoattractant for immune cells via FPR1, and EGFR was found to mediate LL-37-stimulated lung epithelial wound closure and keratinocyte migration. Notably, a GPCR was also found to be involved in lung epithelial wound closure, but it was not FPR1. Involvement of EGFR and GPCRs has also been shown in LL-37 stimu-
ulatory effects on cytokine secretion. In our study, WRW did not significantly block LL-37-mediated cytokine production, whereas AG1478 was highly effective. These data indicate that EGFR, but not FPRL1 is involved in LL-37 modulated cytokine secretion by HCECs. As we did not test the effect of PTX on LL-37-stimulated HCEC cytokine production, we cannot eliminate the possibility that a GPCR other than FPRL1 is involved in this process. The observation that EGFR is involved in LL-37 stimulated cytokine production has also been noted for IL-8 production in lung epithelial cells and keratinocytes. Braff et al. also demonstrated participation of a GPCR in keratinocyte IL-8 production although they suggested this effect was via non-receptor-mediated G-protein activation.

To further investigate the signal transduction pathways involved in mediating LL-37-induced effects on corneal epithelial function, we have tested various intracellular signaling pathways inhibitors on LL-37-stimulated HCEC migration. Of the three MAPK pathways, we have demonstrated that ERK1/2 and c-JNK, but not p38 MAPK, participate in LL-37-induced HCEC migration, as shown by the ability of the respective inhibitors PD98059 and SP203580 to attenuate partially the effect of LL-37. We have also shown that ERK1/2 and c-JNK are involved in LL-37-stimulated cytokine secretion. Previously, LL-37 stimulatory effects on lung epithelial cell proliferation and wound closure, keratinocyte IL-18 secretion, and monocyte activation have been shown to be mediated via ERK1/2 and p38 MAPK. Activation of all three MAPK pathways by LL-37, however, has been reported in airway epithelial cells. Further, we have now demonstrated, for the first time, the involvement of PI3K and TK in LL-37-stimulated HCEC activities. LY294002 appeared to have stronger inhibitory effects than genistein (the TK inhibitor which has also been shown to block EGFR-TK-dependent stimulatory cell effects) as demonstrated by its ability to block LL-37-induced HCEC migration very effectively and to suppress cytokine release by HCECs markedly, suggesting that PI3K must be at the very early stage of the signaling cascades involved in this process. Our data indicate that PKC is not involved in LL-37 induced corneal epithelial cell functions, as an inhibitor (H-7) of this pathway did not significantly influence LL-37-induced cell migration. Taken together, these findings indicate that various intracellular signaling cascades may run parallel or converge in orchestrating the stimulatory effects of LL-37 on corneal epithelial migration (potentially via both FPRL1 and EGFR) and cytokine production (primarily through EGFR signaling).

In summary, our data show that LL-37 is expressed by human corneal epithelium and is upregulated during re-epithelialization. We have shown that LL-37 has modest antimicrobial activity against ocular pathogens in the presence of human tear fluid and stimulates corneal epithelial migration and chemokine/cytokine production via pathways involving FPRL1 and EGFR-TK and TK inhibitors on LL-37 stimulated HCEC cytokine production. ELISA was performed to measure IL-8 (A), IL-6 (B), IL-1β (C), and TNF-α (D) release in cell-free supernatant in the presence and absence of LL-37 (5 μg/mL) from P-HCECs pretreated with or without WRW, AG1478, or genistein. Cells treated with medium alone served as the control. Pretreatment of cells with the antagonist and inhibitors alone had no effect on cytokine secretion (data not shown). Results are expressed as the mean (pg/mg cell protein) ± SEM of one representative experiment repeated two to three times, each performed in triplicate. *P < 0.05 compared to LL-37 alone.
EGFR. These observations indicate that LL-37 potentially stimulates ocular immune defense through direct antimicrobial activity and induction of chemokine/cytokine release and therefore is capable of strengthening both the innate and adaptive immune responses. Furthermore, our data support a role for LL-37 in corneal epithelial wound healing.

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References


