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A multifunctional peptide based on the neutrophil immune defense molecule, CAP37, has antibacterial and wound-healing properties

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ABSTRACT

CAP37, a protein constitutively expressed in human neutrophils and induced in response to infection in corneal epithelial cells, plays a significant role in host defense against infection. Initially identified through its potent bactericidal activity for Gram-negative bacteria, it is now known that CAP37 regulates numerous host cell functions, including corneal epithelial cell chemotaxis. Our long-term goal is to delineate the domains of CAP37 that define these functions and synthesize bioactive peptides for therapeutic use. We report the novel finding of a multifunctional domain between aa 120 and 146. Peptide analogs 120–146 QR, 120–146 QH, 120–146 WR, and 120–146 WH were synthesized and screened for induction of corneal epithelial cell migration by use of the modified Boyden chamber assay, antibacterial activity, and LPS-binding activity. In vivo activity was demonstrated by use of mouse models of sterile and infected corneal wounds. The identity of the amino acid at position 132 (H vs. R) was important for cell migration and in vivo corneal wound healing. All analogs demonstrated antimicrobial activity. However, analogs containing a W at position 131 showed significantly greater antibacterial activity against the Gram-negative pathogen *Pseudomonas aeruginosa*. All analogs bound *P. aeruginosa* LPS. Topical administration of analog 120–146 WH, in addition to accelerating corneal wound healing, effectively cleared a corneal infection as a result of *P. aeruginosa*. In conclusion, we have identified a multifunctional bioactive peptide, based on CAP37, that induces cell migration, possesses antibacterial and LPS-binding activity, and is effective at healing infected and noninfected corneal wounds in vivo. *J. Leukoc. Biol.* 97: 341–350; 2015.

Abbreviations: ATCC = American Type Culture Collection, CAP37 = cationic antimicrobial protein of molecular mass 37 kDa, EU = endotoxin units, H = histidine, HCEC(s) = human corneal epithelial cell(s), LAL = *Limulus* amebocyte lysate, LB = Luria Bertani, MIC = minimum inhibitory concentration, Q = glutamine, R = arginine, RP = reversed-phase, SFM = serum-free medium, TFA = trifluoroacetic acid, W = tryptophan

Introduction

CAP37 [1], also known as azurocidin [2], is heparin binding [3] and was originally discovered in 1984 as a result of its strong antibacterial activity against Gram-negative pathogens, such as *Escherichia coli*, *Salmonella* Typhimurium, and *P. aeruginosa* [4, 5]. However, it is now recognized that CAP37 has more wide-ranging implications in host defense, as in addition to its effect on bacteria, it modulates host cell functions [6]. It is a chemoattractant for monocytes [7], microglia [8], smooth muscle cells [9], and HCEC [10]. It promotes proliferation of smooth muscle cells [9] and HCEC [10] and up-regulates adhesion molecules on endothelium, important for emigration of leukocytes [11]. Furthermore, it is expressed in endothelial and smooth muscle cells associated with atherosclerotic plaques [12] and in the limbal vessels and corneal epithelium in response to ocular infection [13]. Its significance in inflammation and healing has been the focus of our laboratory since its association with inflammatory-mediated diseases [12, 14], endotoxemia [15], and dermal and corneal healing were demonstrated [10, 13, 16].

The analysis of the structural properties of CAP37 has given greater insight into the various functions of this multifunctional innate-immune defense molecule. CAP37 is a 222 aa glycoprotein that consists of 23 positively charged residues, as opposed to 15 acidic residues, making it a highly cationic, basic molecule [17, 18]. Synthetic peptides based on the CAP37 molecule were generated to span the entire 222 aa sequence. Each peptide was ~25 aa long, and the peptides were synthesized with at least 2–5 aa overlapping at each end. Previously, we described a peptide designated 20–44, which was found to have strong antimicrobial activity [19] for Gram-negative pathogens, including *P. aeruginosa* and bound LPS [15]. Published [20] and unpublished data point to a domain that lies between aa 95 and 122, which modulates host cell functions, including protein kinase C activation and cell migration.

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In this study, we identify a domain of the CAP37 protein that lies between aa 120 and 146 and demonstrate for the first time that a synthetic peptide, based on this sequence, has strong antibacterial activity, as well as wound-healing properties. An interesting feature of this stretch of the CAP37 molecule is that in the native neutrophil sequence, the amino acid residue at position 132 is an R [18]. However, in the induced protein in the corneal epithelium, a H replaces this R moiety [13]. The reason and biologic significance for the mutation in the cornea are unknown, and this investigation was performed to determine whether it might have an impact on the function of the protein in ocular inflammation and healing.

Synthetic peptides that comprised the neutrophil and corneal epithelial sequences between aa 120 and 146 were generated. Analogs of these two peptides with various substitutions and replacements of the amino acids were also synthesized to determine the impact on function. We then tested these peptides for antimicrobial, LPS binding, and chemotactic activity, known functions of the CAP37 molecule. To determine if these peptides had bioactivity in vivo, we used an in vivo mouse model of corneal wound healing and *Pseudomonas* bacterial keratitis. Our studies led to the identification of a synthetic peptide based on CAP37 that has significant host-defense activities.

MATERIALS AND METHODS

Synthesis of peptides based on the $CAP37_{120–146}$ sequence

Peptides were synthesized (Peptisyntha, Torrance, CA, USA, and CS Bio, Menlo Park, CA, USA) by use of solid-phase peptide synthesis and fluorenylmethoxycarbonyl chemistry protocols essentially, according to previously described methods [19, 21]. After synthesis, the peptide compound was deprotected and cleaved from the resin by use of a cocktail of TFA-containing scavengers. The resin was filtered off, and the filtrate was evaporated to remove TFA. The peptide compound was precipitated with ether, and the precipitate was filtered off and dried under reduced pressure to obtain the crude peptide, which was then purified by RP-HPLC by use of a Vydac 218TP C18 column (150 × 4.6 mm × 5 μm; Peptisyntha) or a Phenomenex Luna C18 column (250 × 4.6 mm × 5 μm; CS Bio). The mobile phase was a linear gradient of acetonitrile in 0.1% aqueous TFA, and absorbance was monitored at 214 nm. The purified peptides were lyophilized and characterized for purity by analytical RP-HPLC and for integrity and molecular weight by electrospray ionization mass spectrometry, as described previously [19, 21]. Peptide preparations used in this study had <0.05 EU/μg peptide, as determined by the LAL assay (Lonza, Basel, Switzerland).

Production of rCAP37

rCAP37 was produced in human embryonic kidney 293 cells by use of an RSV-PL4 expression vector [8, 11]. The recombinant protein was purified on an HPC4 immunoaffinity column, as described previously [8, 11]. 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 by use of the modified Boyden chemotaxis chamber assay, as published previously [7, 22]. The rCAP37 preparations used in this study had <0.05 EU/μg protein, as determined by the LAL assay (Lonza).

Cell culture

SV40 adenovirus-immortalized HCEC were obtained from Dr. James Chodosh (Boston, MA, USA) and were maintained as described previously [10, 23] in defined keratinocyte SFM (Gibco, Grand Island, NY, USA), containing L-glutamine (2 mM; Gibco), antibiotic-antimycotic (0.1 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, 0.25 μg/ml amphotericin B; Gibco), and growth supplements, as provided by the manufacturer. Primary HCEC were isolated from donor corneas acquired from the Lions Eye Bank (Oklahoma City, OK, USA) and cultured as described previously [23]. All HCEC were starved for 18 h in keratinocyte SFM, without growth factors, before the set-up of the cell migration experiments.

Cell migration

Cell migration was determined by use of the modified Boyden chemotaxis chamber assay, described previously [7, 22, 23]. HCEC were detached by use of 5 mM EDTA in PBS (Gibco) at 37°C for 30 min and adjusted to a concentration of 8×10^5 cells/ml in Gey's buffer (Sigma-Aldrich, St. Louis, MO, USA), containing 0.1% endotoxin-low BSA (Sigma-Aldrich). Polycarbonate polyvinyl pyrrolidone-free filters with 8 μm pores (Whatman, Waltham, MA, USA), coated on the lower side with 50 μg/ml rat-tail collagen I (BD Biosciences, San Jose, CA, USA), separated the upper chamber containing the cells (200 μl 8×10^5 cells/ml) from the lower chamber containing the chemoattractant. rCAP37 at 500 ng/ml was used as the positive control, and the negative control was Gey's buffer containing 0.1% endotoxin-low BSA. After 3 h incubation at 37°C in a 5% CO_2 humidified atmosphere, the filters were removed, stained with Diff-Quick (Dade Behring, Dűdingen, Switzerland), and mounted with Permount (Fisher Scientific, Waltham, MA, USA). Cell migration was determined by counting the number of cells that had migrated through the pores to the opposite side of each filter. Ten adjacent fields were counted/filter under a 40× objective by use of a Nikon Eclipse E200 microscope (Nikon Instruments, Melville NY, USA), and averaged. Cell migration was expressed as fold increase compared with the buffer control, which was arbitrarily defined as one. Chambers were set up in triplicate for each experimental condition.

In vitro bactericidal assay

A single colony of *P. aeruginosa* (#27853; ATCC, Manassas, VA, USA) was grown overnight at 37°C in 5 ml LB broth (Sigma-Aldrich). Overnight culture (100 μl) was subcultured in 5 ml fresh LB broth for 120–150 min at 37°C, with rotation such that the bacterial culture reached log phase. The bactericidal assay was performed essentially as described before [19]. In brief, bacteria were prepared at 10^6 CFU/ml in tryptone saline solution [0.4% tryptone (Difco Laboratories, Detroit, MI, USA), 0.4% NaCl (Fisher Scientific), pH 5.5]. The bacterial suspension (100 μl) was incubated with 100 μl of each peptide diluted in tryptone saline. Reactions were set up in a sterile 96-well tissue-culture plate (Becton Dickinson Labware, Franklin Lakes, NJ, USA), such that the final concentrations of peptides in the wells were 0, 1.25, 2.5, and 5×10^{-5} M (corresponding to 0, 37.5, 75, and 150 μg/ml, respectively). After 3 h incubation at 37°C, serial dilutions of each well were performed and plated on LB agar plates and incubated overnight at 37°C. Bacterial colonies in the absence of peptide were counted at 0 (starting inoculum) and 180 min to assess bacterial growth over the time period of the assay in the absence of the peptide. Bacterial colonies in the presence of the peptide were counted at the end of the 180 min incubation period and the CFU/ml determined. The percent killed in response to the peptides was expressed as a log reduction in CFU/ml. Three independent experiments were performed, with each experimental condition in triplicate.

LPS-binding assay

The in vitro binding of LPS by CAP37-derived peptides was determined as described previously [15, 24]. In brief, increasing concentrations (0.625, 1.25, and 2.5 EU/ml) of LPS from *P. aeruginosa* serotype 10 (Sigma-Aldrich) were incubated with increasing concentrations of peptides (0, 4.2×10^{-6}, 8.4×10^{-6}, and 1.7×10^{-5} M, corresponding to 0, 12.5, 25, and 50 μg/ml, respectively). Reactions were set up in duplicate in a 96-well tissue-culture plate (Becton Dickinson Labware) and incubated for 15 min at room temperature to allow binding of the peptide and inactivation of LPS activity. LPS endotoxin activity was then quantified by use of the LAL chromogenic

assay (Lonza), according to the manufacturer's protocol. The results are expressed as percent inhibition of endotoxin activity, and average ± SEM was calculated from the results of 3 independent experiments.

In vivo corneal wound-healing model

C57BL/6J female mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were acclimated for 1 week and were 8 weeks of age at the start of experiments. All animals were maintained and handled according to institutional guidelines and the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Mice were anesthetized by use of ketamine (100 mg/kg; Bioniche Pharma, Lake Forrest, IL, USA) and xylazine (10 mg/kg; Rompun; Bayer, Shawnee Mission, KS, USA), and the right cornea was wounded as follows: a disposable 2 mm-diameter biopsy punch (Miltex, York, PA, USA) was used to demarcate a circular area on the mouse cornea. The corneal epithelium was carefully removed within the 2 mm-demarcated area with a 0.5 mm burr by use of the Algerbrush II (The Alger Company, Lago Vista, TX, USA). Corneal abrasions were treated at 0 and 16 h with peptides (10^{-8} and 10^{-6} M) or vehicle control (0.9% sodium chloride, pH 5.5; Baxter, Deerfield, IL, USA). Corneal abrasions were visualized by use of sterile fluorescein sodium ophthalmic strips United States Pharmacopeia (Fluorets; Laboratoire Chauvin, Aubenas, France), dampened with sterile PBS. Images were taken at 0, 16, and 24 h immediately following fluorescein staining by use of a surgical microscope equipped with a camera (OPMI VISU 140; Carl Zeiss Surgical, Oberkochen, Germany). The areas of the open wounds were quantified by use of ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA), and the measured area of each wound was expressed as a percentage of the starting area for this particular wound. The results were then reported as percent wound closure and averages ± SEM of at least 6 mice/group were plotted.

In vivo bactericidal activity

A circular wound was created on the mouse cornea by removing the epithelium, as described above. While the mice remained under anesthesia, the wounds were infected with 10^5 CFU *P. aeruginosa* (ATCC #27853) in 10 μl saline (0.9% sodium chloride, pH 5.5; Baxter), prepared from an overnight culture in LB. The bacterial culture remained on the abraded cornea for 1 h to allow the infection to occur. The excess bacteria were then washed away with 1 ml saline, and treatments were started. The peptide treatments (0, 6.7×10^{-4}, 1.67×10^{-3}, 3.35×10^{-3}, and 6.7×10^{-3} M, corresponding to 0, 2, 5, 10, and 20 mg/ml, respectively) were administered in 10 μl saline every 30 min for 6 h on d 1. Infected wounds were treated twice on d 2 (at 22 and 28 h postinfection) and once on d 3 (at 46 h postinfection). Mice were euthanized at 48 h postinfection. Whole eyes were enucleated and each placed in 400 μl sterile PBS in 2 ml microtubes (Sarstedt AG, Nümbrecht, Germany). Sterile, 1 mm glass beads (BioSpec Products, Bartlesville, OK, USA) were added, and the tubes were processed for 1 min in an Omni Bead Ruptor 24 (OMNI International, Kennesaw, GA, USA) at 5.65 m/s to homogenize the eye globes. The number of CFU/eye was quantified by plating serial dilutions of each homogenate by use of a method described previously [25]. Individual results from each mouse and the means from 5 mice/group were graphed and analyzed.

Peptide cytotoxicity

A 96-well tissue-culture plate (BD Falcon, BD Biosciences) was seeded with 3×10^4 HCEC/well, and cytotoxicity of the peptides was tested the next day by use of in situ Trypan blue staining, as described in ref. [26]. In brief, the adherent HCEC were washed once with vehicle and then incubated with vehicle containing peptide. At the end of the incubation time, cells were washed with PBS, 3 times to remove the peptide, and incubated with Trypan blue 0.2% diluted in PBS for 1 min at 22°C. Cells were then washed once with PBS to remove excess Trypan blue and fixed immediately with 1% formaldehyde diluted in PBS for 10 min at 22°C. Cells were washed once in PBS, and pictures were taken by use of an inverted microscope equipped with a digital camera (Nikon Eclipse TS100; Nikon Instruments). The peptide concentrations tested were 0, 6.7×10^{-5}, 6.7×10^{-4}, 1.67×10^{-3}, 3.35×10^{-3}, and 6.7×10^{-3} M, and a time-course was performed by incubating the cells with 6.7×10^{-3} M of peptide 120–146 WH for 0, 5, 15, and 30 min.

Statistical analysis

GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA) was used for statistical analysis of the results. For in vitro results, multiple comparisons (each group was compared with the control group in the chemotaxis and bactericidal experiments) were performed by use of a one-way ANOVA, followed by a Dunnett's multiple comparisons test. For in vivo results, an unpaired nonparametric *t*-test (Mann-Whitney) was performed to compare each treatment group with the control.

RESULTS

Characterization of purified peptides

Peptide purity, as assessed by RP-HPLC, was between 93.3% and 99.6% on all preparations. Four peptides were synthesized based on the amino acid sequence of CAP37 between residues 120 and 140 (**Fig. 1A**). These included peptide 120–146 QR (GTRCQVAGWGSQRSGGRLSRFPRFVNV), consisting of residues 120–146 of the native neutrophil-derived CAP37 sequence [17, 18], and peptide 120–146 QH (GTRCQVAGWGSQHSGGRLSRFPRFVNV), based on aa residues 120–146 of the corneal epithelial-derived CAP37 [13] (Fig. 1B). The 2 peptides are identical, except for the residue at position 132, which is an R in the neutrophil-derived sequence and a H in the corneal epithelial-derived sequence.

To determine whether changing the amino acid residue at position 131 would have a bearing on function, we synthesized 2 analogs of these parent peptides by substituting a W residue for the Q residue. These 2 peptides are designated 120–146 WR (GTRCQVAGWGSWRSGGRLSRFPRFVNV) and 120–146 WH (GTRCQVAGWGSWHSGGRLSRFPRFVNV; Fig. 1B). Molecular weights of all 4 peptides were determined by electrospray ionization mass spectrometry and were determined as 2980 Da for 120–146 QR, 2960 Da for 120–146 QH, 3038 Da for 120–146 WR, and 3018 Da for 120–146 WH. Residues 120–146 are partially exposed in the native CAP37 protein but are also part of one of the β-barrels found in CAP37 (Fig. 1C).

CAP37 peptides 120–146 QH and 120–146 WH promote migration of HCEC

As the native CAP37 molecule is known to have chemotactic activity for HCEC [10, 23], we queried whether peptides based on the neutrophil-derived protein and the corneal epithelial-derived peptide would display this activity. Peptides 120–146 QR and 120–146 QH were tested for their effect on migration of SV40-immortalized HCEC (**Fig. 2A**) and primary HCEC (Fig. 2B). Figure 2A shows that peptide 120–146 QH significantly induced migration of HCEC at concentrations between 10^{-10} and 10^{-6} M. Peptide 120–146 QR, based on the native neutrophil-derived sequence, has no significant activity on HCEC at the concentrations tested. Peptide 120–146 QH at 10^{-6} M has comparable levels of activity with rCAP37, the positive control included in these assays. The migration of HCEC responded with a bell-shaped dose-response, typically associated with chemotactic agents [10].

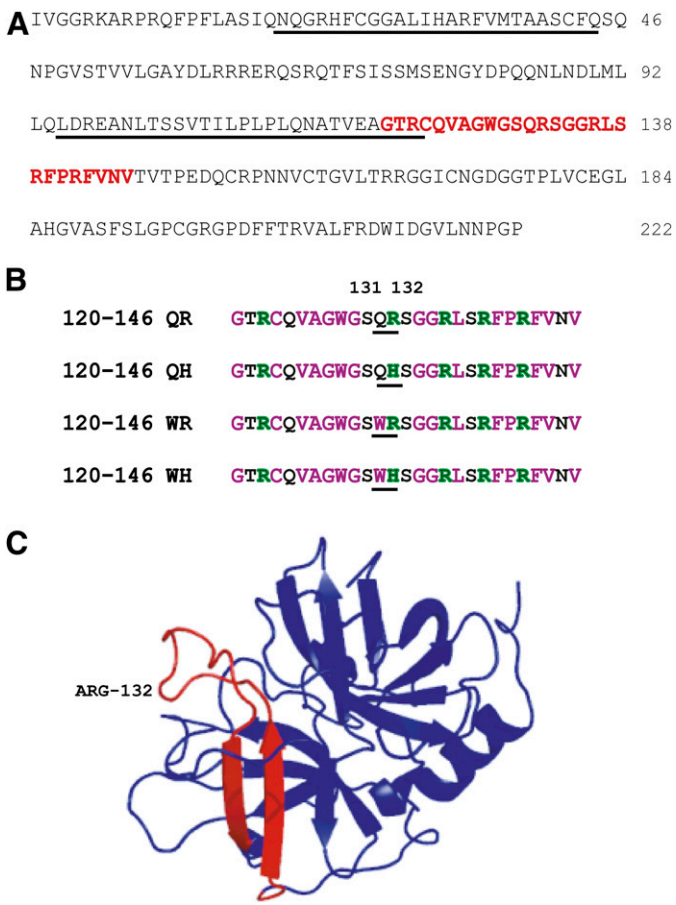

Figure 1. CAP37-derived peptide 120–146 and analogs. (A) The primary sequence of human neutrophil CAP37 is shown, and the known functional domains of the protein are underlined. The 1st underlined domain corresponds to peptide 20–44, which has bactericidal and LPS-binding activities. The 2nd underlined domain corresponds to peptide 95–122, which activates protein kinase C in endothelial cells. The peptide 120–146 described in this study is shown in red. (B) Sequence alignment of the 4 synthetic CAP37 peptides. Sequence based on the native neutrophil CAP37 (120–146 QR), corneal epithelial CAP37 (120–146 QH), and the modified peptides with the change in the Q residue at position 131 to a W residue (120–146 WR and 120–146 WH). Amino acid replacements at positions 131 and 132 are underlined. This peptide and analogs comprise a mixture of positively charged (indicated in green) and hydrophobic (indicated in purple) residues. (C) Domain 120–146 is indicated in red on the 3-dimensional ribbon diagram representation of CAP37 and consists of residues located on surface loops, including R (ARG)-132 (present in the neutrophil-derived CAP37) and residues forming β-sheets that are partly nested inside of the protein. The R at position 132 is replaced with a H in the corneal epithelial-derived CAP37.

To ensure that these findings were applicable to primary HCEC, the study was repeated by use of corneal epithelial cells derived from 4 individual human donor corneas. Data shown in Fig. 2B confirm the differential activities of the 2 peptides. These findings demonstrate that the substitution of residue R132 to H132 significantly increased the effect of the peptide on cell migration and that peptide 120–146 QH induces HCEC migration. There was variation in the extent of migration displayed by the individual donor cells, and 1 donor showed no dose response to the peptide (data not included in this composite figure).

To determine the impact of the replacement of residue Q131 by W131 on migration, we studied the effect of peptide 120–146 WH on the SV40-transfected HCEC cell line by use of the modified Boyden chamber assay at concentrations ranging from 10^{-10} to 10^{-4} M. As will be noticed (Fig. 2A), the peptide 120–146 WH was not as potent as 120–146 QH, as migration was seen only at the higher concentrations of 10^{-6} and 10^{-4} M, although it should be noted that the maximum fold increase in migration was comparable with the levels of HCEC migration obtained with 120–146 QH.

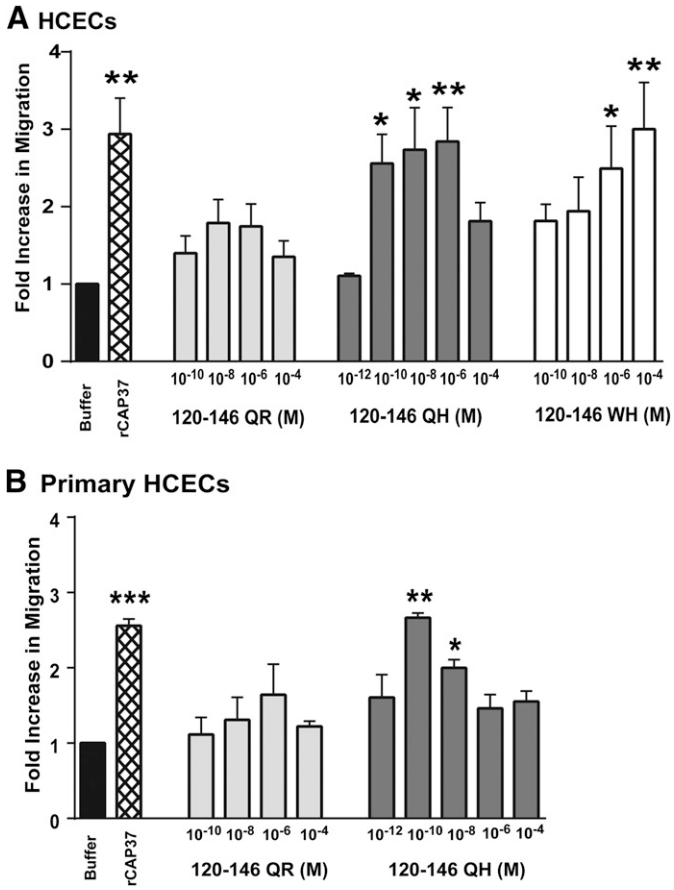

Figure 2. The peptide 120–146 induces cell migration. Migration of (A) immortalized HCEC and (B) primary HCEC was determined by the modified Boyden chemotaxis chamber method in response to buffer control (0.1% BSA in Gey's buffer), rCAP37 (1.7×10^{-8} M or 500 ng/ml), 120–146 QR (10^{-10} M, 10^{-8} M, 10^{-6} M, 10^{-4} M), 120–146 QH (10^{-12} M, 10^{-10} M, 10^{-8} M, 10^{-6} M, 10^{-4} M), and 120–146 WH (10^{-10}, 10^{-8}, 10^{-6}, 10^{-4} M; in A only). The range of peptide concentration tested was 10^{-12} to 10^{-4} M, which corresponds to 3 pg–300 μg/ml. The average ± SEM number of migrated cells in the buffer control was 21 ± 9. For each independent experiment, the buffer control was arbitrarily assigned the value of 1, and cell migration was expressed as fold increase over the buffer control. Each test point was run in triplicate. Results from between 3 and 6 independent experiments are shown. Results are expressed as mean ± SEM. Each treatment was compared with the buffer control by use of a one-way ANOVA, followed by a Dunnett's multiple comparisons test by use of GraphPad Prism. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

Substitution of residue Q131 with hydrophobic W131 enhances bactericidal activity

To determine whether peptides based on the sequence 120–146 could have additional activities, we tested their bactericidal activity in vitro, as it is known that the native protein is strongly bactericidal for Gram-negative pathogens [5, 6]. **Figure 3** demonstrates the bactericidal activity of the 4 CAP37-derived analogs against *P. aeruginosa*. Peptides 120–146 QR and 120–146 QH demonstrate activity against *P. aeruginosa* at the highest concentration tested (5×10^{-5} M or 150 μg/ml; Fig. 3). These peptides demonstrate 1–2 log reduction in CFU/ml, reducing the starting CFU from $\sim 5 \times 10^5$ CFU/ml to 5×10^3 CFU/ml with peptide 120–146 QR and to 5×10^4 CFU/ml with peptide 120–146 QH. Peptide 120–146 QR, based on the neutrophil-derived sequence, appears to be marginally more active against *P. aeruginosa* than the corneal-derived peptide, although this difference is not statistically significant ($P > 0.05$). This leads us to infer that the identity (R vs. H) of the amino acid at position 132 did not alter the bactericidal effect of the peptide. It will also be apparent that in the absence of the peptide, there is an increase of ~1 log in bacterial growth during the 180 min incubation period (from 5×10^5 to $\sim 5 \times 10^6$ CFU/ml).

By contrast, the substitution of the hydrophilic Q residue at position 131 with a hydrophobic W residue (W131) increased the bactericidal effect of the peptide at all 3 doses tested. Peptide 120–146 WR results in a 2 log reduction of the starting concentration of bacteria at the lowest dose of peptide (1.25×10^{-5} M or 37.5 μg/ml), and both the intermediate and highest doses of peptide (2.5 and 5×10^{-5} M corresponding to 75 and 150 μg/ml, respectively) lead to a 5.5 log reduction or killing of 100% of the bacteria (Fig. 3). Similar activity is obtained with peptide 120–146 WH (Fig. 3). Taken together, our in vitro results indicate that the most potent antimicrobial analogs are 120–146 WR and 120–146 WH. Gentamicin at 0.84×10^{-5} M (4 μg/ml, 2× MIC), which is at approximately a similar molar concentration as the peptide at 1.25×10^{-5} M (37.5 μg/ml) is not as effective as peptides 120–146 WR and 120–146 WH under these in vitro killing conditions.

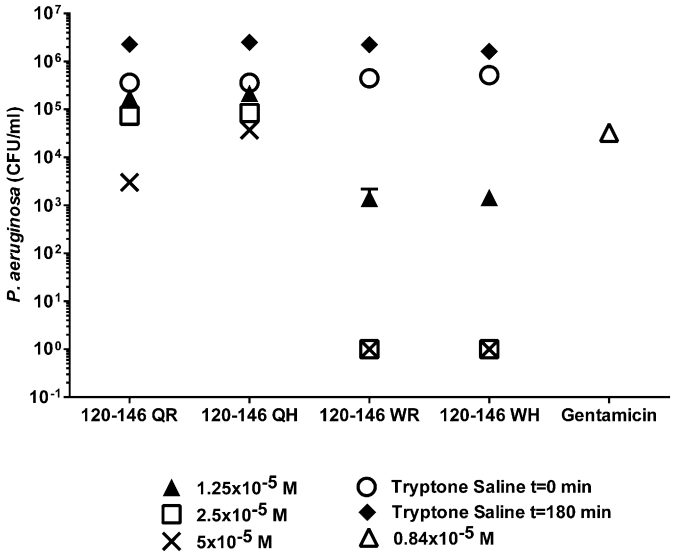

Figure 3. The peptide 120–146 is bactericidal, and substitution of residue Q131 to W131 enhances its bactericidal activity. *P. aeruginosa* at 5×10^5 CFU/ml were incubated in vitro in tryptone saline solution or with peptide 120–146 QR, 120–146 QH, 120–146 WR, or 120–146 WH or gentamicin in tryptone saline. The range of peptide concentration tested was 1.25×10^{-5}–5×10^{-5} M, which corresponds to 37.5–150 μg/ml. Gentamicin was used at 0.84×10^{-5} M (corresponding to 4 μg/ml), which is twice its MIC and approximately equivalent to the lowest peptide concentration used. Bacteria were quantified, and the log CFU/ml at the start (t = 0 min) and end of the experiment (t = 180 min) are indicated by the open circle and closed diamond, respectively. Each experimental condition was performed in triplicate, and average results from 3 independent experiments are plotted ± SEM on a logarithmic scale. Each treatment was compared with the tryptone saline control by use of a one-way ANOVA, followed by a multiple comparisons test in GraphPad Prism. The 4 peptides at all concentrations tested, as well as gentamicin, had highly significant killing activities ($P < 0.0001$) when compared with the tryptone saline control at t = 180 min.

CAP37-derived analogs of peptide 120–146 bind LPS

All 4 peptides bound *P. aeruginosa* LPS (0.625–2.5 EU/ml) in a dose-dependent manner (**Fig. 4A–D**). The highest concentration of all 4 peptides (1.7×10^{-5} M or 50 μg/ml) almost completely abolished the capacity of LPS to cleave and activate a proenzyme in the LAL, thus inhibiting the conversion of the substrate to a chromogenic product. The flat line obtained with peptide 120–146 WR (Fig. 4C) at the lowest concentration of peptide (4.2×10^{-6} M or 12.5 μg/ml) would suggest that peptide 120–146 WR has the strongest LPS-binding capacity.

Peptide 120–146 WH promotes wound healing in a mouse corneal abrasion model

With the use of an in vivo mouse model of corneal wound healing, we explored the effect of topical application of peptides 120–146 WR and 120–146 WH at 10^{-6} and 10^{-8} M in comparison with vehicle (saline). We compared the rates of closure at 16 h and 24 h (**Fig. 5**). By 16 h, peptide 120–146 WH at both concentrations tested had effectively reduced the size of the wound by ~80%. This was significantly greater than the rate of closure in response to the vehicle ($P < 0.01$). By 24 h, the peptide at 10^{-6} M had effectively closed almost 90% of the surface area ($P < 0.001$ compared with saline). By contrast, there was no significant acceleration of wound healing when comparing analog 120–146 WR with the vehicle control (Fig. 5A, left). This suggests that the H in position 132 is important for effective corneal wound healing in vivo. Representative images of fluorescein-stained wounds are shown in Fig. 5B and illustrate the smaller size of the wounds treated with the peptide compared with the vehicle-treated wounds.

Peptides 120–146 WR and 120–146 WH have antimicrobial activity in a mouse model of *Pseudomonas* keratitis

Based on the data obtained from in vitro bactericidal studies in Fig. 3 and the in vivo wound-healing studies shown in Fig. 5, we selected peptides 120–146 WR and 120–146 WH for further in vivo analysis. In this set of experiments, we investigated the

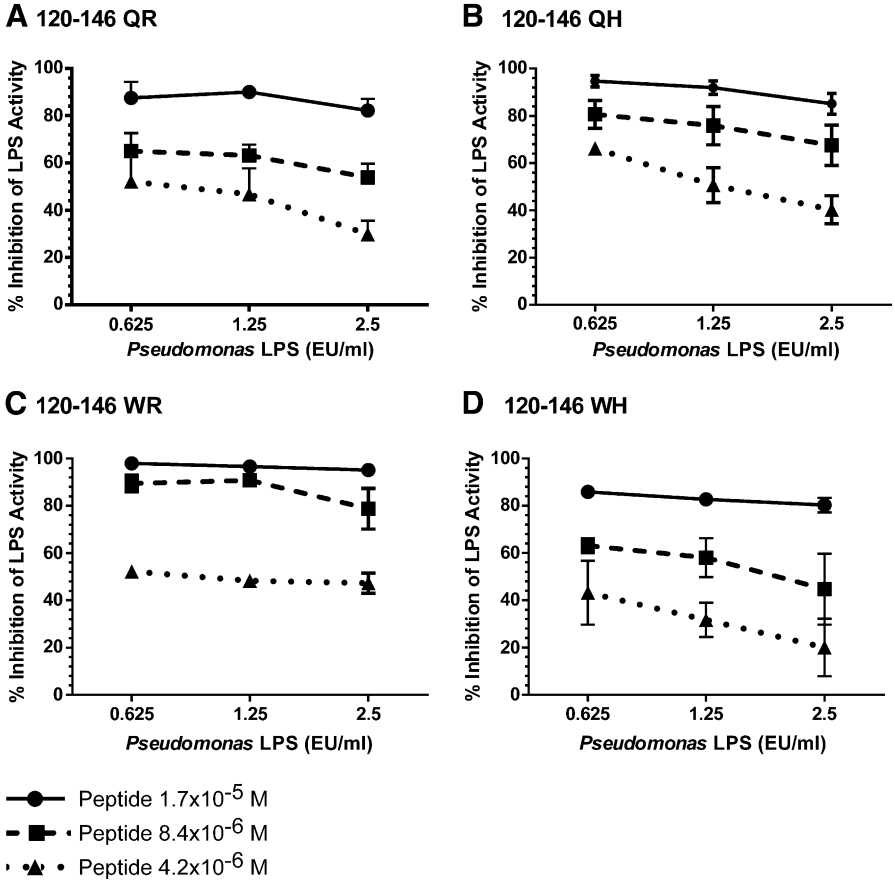

Figure 4. CAP37-derived peptide analogs inhibit LPS activity. Indicated concentrations of *Pseudomonas* LPS were incubated with peptides 120–146 QR (A), 120–146 QH (B), 120–146 WR (C), or 120–146 WH (D) for 15 min at room temperature, and LPS activities were then determined by the LAL assay. The range of peptide concentration tested is 4.2×10^{-6} to 1.7×10^{-5} M, which corresponds to 12.5–50 μg/ml. Each experimental condition was performed in duplicate, and 3 independent experiments were performed for each peptide. Results are expressed as percent inhibition of LPS activity, and average ± SEM was plotted.

efficacy of the peptides in a mouse model of *Pseudomonas* keratitis. Corneal wounds were created as described, and a suspension of *P. aeruginosa* was immediately applied to the wounds. The bacteria were left undisturbed on the surface of the cornea for 1 h to provide sufficient time for infection of the cornea to occur. Thereafter, the excess bacteria were washed away. Wounds were treated with the peptides solubilized in saline (vehicle) and vehicle alone. As shown in **Fig. 6**, the CFU/eye in the untreated animals was between 5×10^5 and 5×10^6. Treatment with peptide 120–146 WR (Fig. 6A) showed a significant ($P < 0.01$) decrease in CFU/eye, resulting in a mean reduction of almost 2 logs with the highest dose of peptide used. Peptide 120–146 WH was highly effective in reducing the number of CFU isolated from each eye. All 4 mice in the group treated with 6.7×10^{-3} M (20 mg/ml) showed a 6 log reduction or complete sterilization of the eye (Fig. 6B). Three of the 5 mice receiving the 3.35×10^{-3} M (10 mg/ml) dose also showed the absence of *Pseudomonas* growth. Peptide 120–146 WH appears to be more effective than 120–146 WR in vivo, whereas both peptides appeared equivalent in the in vitro bactericidal assay (Fig. 3).

In vitro cytotoxicity

As the concentration of the peptides that resulted in the most effective clearance of the bacterial infection in the in vivo studies was relatively high ($3.35–6.7 \times 10^{-3}$ M), we tested the cytotoxicity of the 4 peptides on HCEC monolayers (**Fig. 7**). The cytotoxicity of the peptides is dose and time dependent. All 4 peptides showed minimal in vitro cytotoxicity with the 2 lowest concentrations (0.067 and 0.67×10^{-3} M) used, and cell viability was >90%. In general, peptides 120–146 QR and 120–146 QH showed less cytotoxicity than peptides 120–146 WR and 120–146 WH, indicating that the alteration from the Q to W impacted the host cell viability. Almost 60% cell death was obtained in vitro with the highest dose of peptide 120–146 WH (6.7×10^{-3} M), which was shown to eliminate 100% of the bacterial infection in vivo (Fig. 7A). We measured the time-course of cell viability of this dose of peptide 120–146 WH in vitro and determined a linear relationship between the time of exposure and the cytotoxicity (data not shown). Cell viability at 5 min was almost 90%, at 15 min, it was 70%, and at 30 min, which is the time-point represented in Fig. 7A, the viability was reduced to 40%. It is difficult to ascertain the exact time of exposure of the corneal epithelium in vivo, as once the drop is delivered to the eye, it is likely diluted by blinking and tears and is absorbed or dispersed through the stroma. However, it is likely that the peptide stays in direct contact with the cornea at the delivered dose for <30 min. According to our studies, decreasing the concentration of peptide up to 50% (3.35×10^{-3} M) greatly increased viability of host cells from 40% to 80%, while significantly decreasing the bacterial infection.

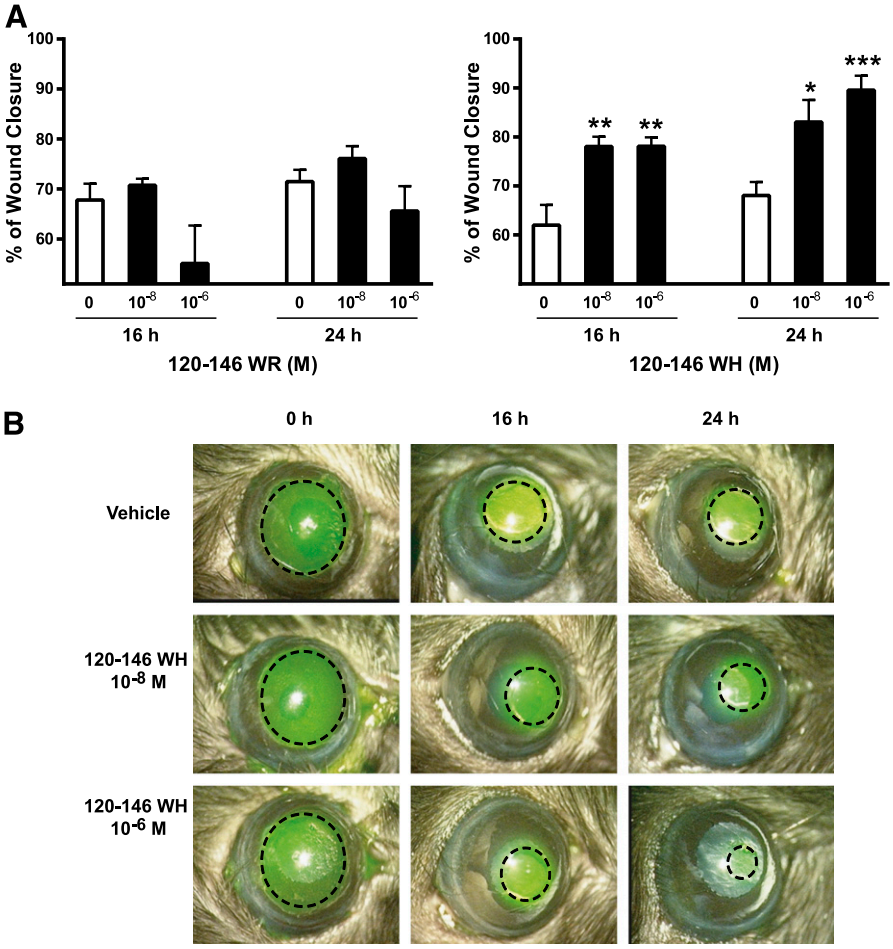

Figure 5. Peptide 120–146 WH promotes corneal wound healing. A circular wound was created on the mouse cornea by removing the epithelium. Wounds were treated at 0 h and 16 h with indicated concentrations of peptide 120–146 WR or 120–146 WH dissolved in 0.9% sodium chloride. The range of peptide concentration tested is 10^{-8} to 10^{-6} M, which corresponds to 30 ng–3 μg/ml. Wounds were visualized by fluorescein staining and measured at 0, 16, and 24 h. (A) Data are represented as the percentage of wound closure at 16 h and 24 h and are expressed as average ± SEM from at least 6 mice/group. Each group was compared with the control group (0.9% sodium chloride; no peptide) by use of the nonparametric and unpaired Mann-Whitney statistical test in GraphPad Prism. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. (B) Representative images are shown for the active peptide 120–146 WH and saline vehicle at 0, 16, and 24 h following treatment. The dotted lines are included to help visualize the edge of the wounds.

DISCUSSION

Our long-term goal is to delineate the domains of CAP37 responsible for the various functions of the protein and to synthesize bioactive peptides for therapeutic use. In this study, we report the novel finding of a multifunctional peptide based on aa 120–146 (Fig. 1A–C). As summarized in **Table 1**, this peptide combines induction of cell migration (Fig. 2) and bactericidal (Fig. 3) and LPS-binding (Fig. 4) activity. We have previously reported on a CAP37-derived bioactive peptide based on aa 20–44 (Fig. 1A) that has bactericidal and LPS-inactivating potential [15, 19, 24]. However, unlike peptides based on 120–146, it did not induce migration of host cells.

When we discovered that the corneal epithelial-derived CAP37 had a single variation at position 132 [13], we wanted to investigate what physiologic role this might play in ocular defense against infection and in the corneal immune response. Figure 2 demonstrates an apparent differential activity of the neutrophil sequence (120–146 QR) and the corneal epithelial sequence (120–146 QH) on the migration of corneal epithelial cells, an indication, perhaps, that the presence of the H residue enhances interaction with a binding site on the corneal epithelial cells. Further manipulation of the peptide 120–146 QH by changing the Q residue at position 131 with a W residue (120–146 WH) did not affect migration significantly. However, this change from a Q to a W did lead to a significant increase in the bactericidal activity of the peptides (Fig. 3). This particular change from a hydrophilic to a hydrophobic residue provides for a potentially dramatic change in the confirmation of these peptides, altering activity. Furthermore, the combination of a W in position 131 with the H in position 132 (120–146 WH) resulted in a peptide that was effective in accelerating corneal wound healing in vivo. The H at position 132 appears to be critical for this host cell effect, as a peptide with an identical sequence, except for the replacement by an R residue (120–146 WR), was unable to promote wound healing.

These peptides probably do not assume their native conformation in solution, but modeling studies carried out on residues 120–146, based on the crystal structure of the native protein from neutrophils, suggest that these peptides retain much of the quasi-native-closed form of the loop as a result of the likely association of the 2 hydrophobic arms to form a stem so as to reduce the entropic cost of hydrating all of the nonpolar side chains. The hydrophobic nature of the W at position 131 in 120–146 WH most likely results in tighter loop conformation as the hydrophobic residue becomes buried in the center of the peptide. This lends to the fact that the mutation of this residue is important when considering that 120–146 WH is active, whereas 120–146 WR is not effective in wound closure and

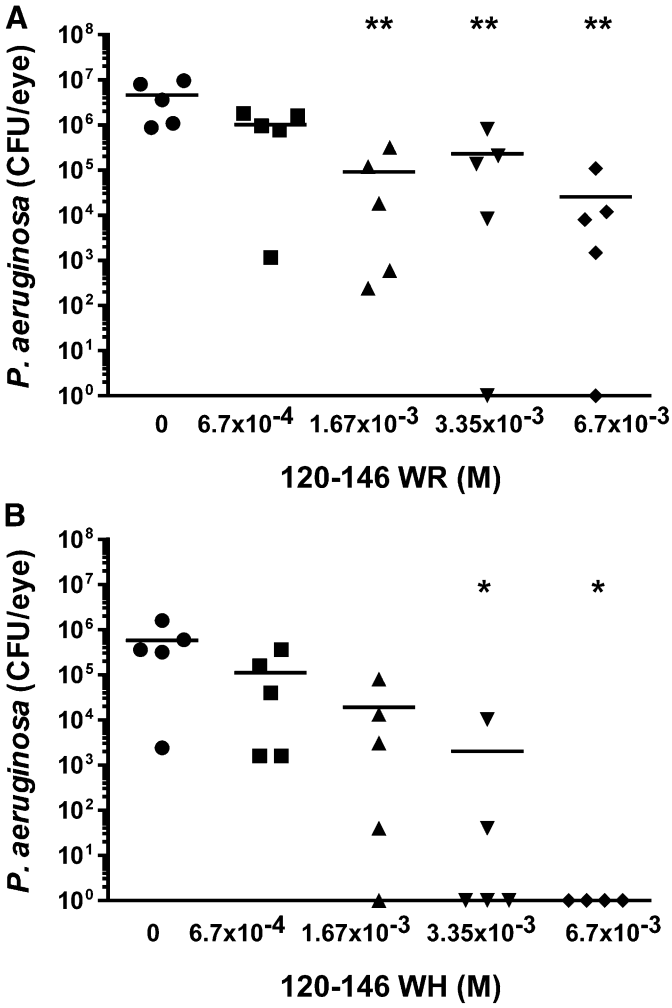

Figure 6. Peptides 120–146 WR and 120–146 WH have antimicrobial activity in a mouse model of *Pseudomonas* keratitis. The mouse corneal epithelium was removed by abrasion to create a circular wound, as described in Materials and Methods. Each wound was infected with 10^5 CFU of *P. aeruginosa* and treated with 0, 6.7×10^{-4}, 1.67×10^{-3}, 3.35×10^{-3}, and 6.7×10^{-3} M, corresponding to 0, 2, 5, 10, and 20 mg/ml, respectively, of peptide 120–146 WR or 120–146 WH, dissolved in 0.9% sodium chloride. Each peptide treatment was administered topically in a volume of 10 μl, according to the regimen described in Materials and Methods. Mice were euthanized at 48 h postinfection, and the eyes were collected, and homogenized. The number of CFU was quantified for each eye by plating serial dilutions of eye homogenates. Results from each mouse and averages from 5 mice/group were plotted. Each group was compared with the control group (0.9% sodium chloride; no peptide) by use of the nonparametric and unpaired Mann-Whitney statistical test in GraphPad Prism. $*P < 0.05$, and $**P < 0.01$.

re-epithelialization. The H at position 132 potentially affects the entire conformation and charge of the peptide. Although H and R residues at position 132 are hydrophilic, charged residues, the structure of the H moiety results in a conformation of the peptide that is capable of interacting with a binding site on the epithelial cells. Further studies will determine the activity of these peptides on monocyte chemotaxis and their role in dermal wound healing.

In addition to its ability to promote re-epithelialization and healing in a corneal abrasion model (Fig. 5), peptide 120–146 WH was effective in clearing a *P. aeruginosa* corneal infection in vivo (Fig. 6). Our intent in this bacterial keratitis study was to focus on the ability of the peptides to clear a bacterial infection, and thus, we used an inoculum of *P. aeruginosa* that was substantial. The starting inoculum in the absence of the peptide increased to levels of 1×10^6 to 1×10^7 CFU/eye. The ability to clear these many bacteria from the eye is significant. Although we did not perform histologic or visual function studies, we saw no gross morphologic differences between the vehicle- and peptide-treated eyes. Future studies are planned to optimize the therapeutic dose of peptide by investigating the most effective dosing regimen and formulation that will lead to effective clearance of the infection, while minimizing cytotoxicity on host cells. Upcoming studies will also explore the mechanism of bacterial clearance further by determining the effect of the peptide on levels of inflammation, identity of migrating leukocytes, up-regulation of cytokines, and other immune-mediated responses and include testing of the visual function. Peptide 120–146 WR was also effective in clearing the infection, but as this peptide was not effective in the wound-healing model, it would appear that peptide 120–146 WH has the strongest potential for further development (Table 1). The concentrations of peptides that are effective in clearing bacteria $3.35–6.7 \times 10^{-3}$ M are greater than those used in the noninfected wound-healing experiments (10^{-6} M). As indicated, our future studies will involve the titration of the dosing and concentration of the peptides to be able to achieve a therapeutic dose that will accomplish both functions.

As far as we are aware, there are currently no therapeutics on the market that combine wound healing with antibiotic properties; therefore, the multifunctionality of peptide 120–146 WH makes it a strong candidate for use as a therapeutic for treating infections of the cornea as a result of *Pseudomonas*, as well as noninfected corneal wounds. These findings are clearly the first step in this process, and future studies will entail the development of appropriate formulation, safety pharmacology, and toxicology studies to establish whether a bioactive peptide, based on the sequence 120–146 WH, can be developed as a topical ophthalmic treatment.

Approximately 30,000 cases of microbial keratitis are reported annually in the United States [27]. Bacterial keratitis is most often a result of the microorganisms *Staphylococcus aureus* and *P. aeruginosa* in the United States [28]. Infections as a result of *P. aeruginosa* collectively account to 37% of visual impairment cases in North America and are most frequently seen in those who use extended-wear contact lenses, immunocompromised individuals, those with invasive corneal injuries, or individuals who have undergone incisional refractive surgery [29]. Bacterial keratitis caused by *P. aeruginosa* can leave an individual blind in <36 h. Existing treatments are not always effective, as bacterial resistance has developed rapidly to current topical antibiotics. An innovative feature of antimicrobial peptides is that their novel mechanism of action has the potential to circumvent the development of bacterial-resistance mechanisms. Their amphipathic nature allows them to intercalate within, bind to, and disrupt the bacterial membranes by creating pores that disrupt

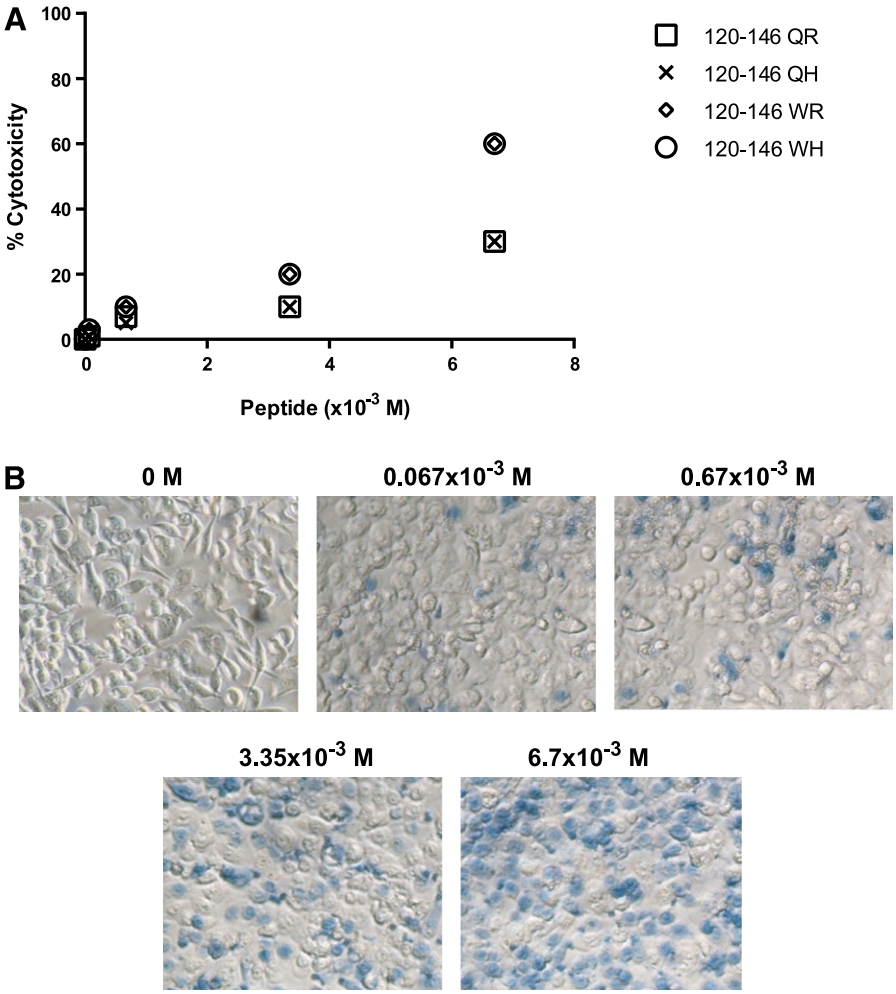

Figure 7. In vitro cytotoxicity of CAP37-derived peptide 120–146 and analogs on HCEC. Cytotoxicity on immortalized HCEC was determined by in situ Trypan blue staining of HCEC monolayers in response to peptide. (A) Dose-response study performed with peptides 120–146 QR, 120–146 QH, 120–146 WR, and 120–146 WH at 0, 0.067, 0.67, 3.35, and 6.7×10^{-3} M, incubated with the cells for 30 min. Results were obtained by counting the number of dead cells within a field of 50 consecutive cells. This was repeated on 3 different fields for each experimental condition. The average of each triplicate was plotted. (B) Representative pictures of Trypan blue staining are shown to illustrate cell viability in response to peptide 120–146 WH at concentrations of 0, 0.067, 0.67, 3.35, and 6.7×10^{-3} M. Percent cytotoxicity was determined as ~0, 3, 10, 20, and 60%, respectively.

membrane proteins, ion gradients, and membrane permeability [30, 31], leading to the death of the microorganism. Our priorities are now to determine the efficiency of peptide 120–146 WH against Gram-negative clinical isolates that are resistant or multiresistant to existing topical ophthalmic antibiotics. The focus remains on Gram-negative bacteria, as the native CAP37 does not display strong bactericidal activity against Gram-positive organisms.

Ocular wound healing is a complex process involving a number of steps that include cell migration, proliferation, cell-matrix adhesion, and tissue remodeling. We had shown previously that CAP37 promoted HCEC migration and proliferation in vitro [10] and that it up-regulated the expression of integrins $\alpha 3$ and $\beta 1$, 3 key components in the wound-healing process. This led us to hypothesize that CAP37 may play a role in corneal wound healing. Given the limitations of use of a whole protein as a therapeutic, we believed that structure-function studies would permit us to identify peptides, such as 120–146 WH, with a defined function that could specifically accelerate and aid in wound healing. A peptide with dual therapeutic properties, possessing both bactericidal and wound-healing effects, would be highly desirable to treat corneal infections, as bacterial keratitis is usually associated with a wound or at least a breach in the corneal epithelium integrity. The analog 120–146 WH is our best candidate for such dual activity, based on our findings that it has robust killing activity and wound-healing properties in vivo.

TABLE 1. Summary of biologic activities of peptides tested in this study

Peptide	HCEC chemotaxis	In vitro killing	LPS binding	In vivo wound healing	In vivo killing
120–146 QR	−	+	+++	ND	ND
120–146 QH	++	+	+++	ND	ND
120–146 WR	ND	+++	+++	−	+
120–146 WH	+	+++	+++	+++	+++

The number of + signs is commensurate with the level of activity; −, no activity; ND, not determined.

AUTHORSHIP

A.K.-J. performed the in vivo studies, contributed to the in vitro studies, and wrote and edited the manuscript. S.N.-M. performed the in vitro bactericidal experiments, contributed to the LPS-binding and in vivo studies, prepared the figures, and edited the manuscript. G.L.G performed the in vitro chemotaxis and in vivo wound-healing studies and contributed to the writing of the manuscript. H.H. and L.M. contributed to the LPS-binding studies. H.A.P. designed the project, analyzed the data, and wrote and edited the manuscript. All authors have read, commented on, and approved the submission of the manuscript.

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DISCLOSURES

S.N.-M., H.H., and L.M. have no financial or commercial conflicts of interest. G.L.G., A.K.-J., and H.A.P. are coinventors on a patent entitled, "Peptide compounds and methods of production and use thereof," filed with the United States Patent and Trademark Office on December 3, 2013.

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antimicrobial peptide · cell migration · corneal wound healing · *Pseudomonas* · keratitis