Corneal Expression of the Inflammatory Mediator CAP37

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Purpose. CAP37 is a polymorphonuclear neutrophil (PMN)–derived inflammatory protein with potent antibiotic and chemotactic activity. To further investigate the biological significance of CAP37 in infection and inflammation, a well-characterized in vivo rabbit model of bacterial keratitis was selected to study its contribution to host defenses.

Methods. One hundred colony-forming units of log phase *Staphylococcus aureus* was injected intrastromally. Eyes were enucleated at 5 to 25 hours after infection and CAP37 detected by immunohistochemistry. To identify the mechanism of CAP37 upregulation in corneal epithelium, in vitro studies using immortalized human corneal epithelial cells (HCECs) were undertaken to determine whether proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), induce CAP37. Because adhesion of leukocytes is important in leukocyte–epithelium interactions, the effect of CAP37 on expression of intercellular adhesion molecule (ICAM)-1 on HCECs was determined by flow cytometry.

Results. Strong staining for CAP37 was demonstrated in the corneal epithelium, stromal fibroblasts, ciliary epithelium, related limbus, ciliary vascular endothelium, and bulbar conjunctiva in rabbits injected with *S. aureus*. The most dramatic expression of CAP37 aside from that in the PMNs occurred in the corneal epithelium. The in vitro studies suggest that CAP37 induction is regulated by TNF-α and IL-1β. In addition, ICAM-1 expression on HCECs was increased in response to CAP37. Molecular cloning of corneal epithelial CAP37 indicated strong sequence identity with an extensive region of PMN-CAP37.

Conclusions. The findings in this study describe the extraneutrophilic expression of CAP37 in response to infection and suggest a role for CAP37 in host defense against infection in the eye. (*Invest Ophthalmol Vis Sci.* 2002;43:1414–1421)

Bacterial keratitis is a common clinical problem and an important component of many ocular infections, especially among those who have sustained penetrating corneal injuries, have used extended-wear contact lenses, have undergone incisional refractive surgery, or are immunocompromised.[1–4] Common causative organisms are the Gram-positive pathogen *Staphylococcus aureus* and the Gram-negative bacterium *Pseudomonas aeruginosa*.[5–7] The bacterial products and toxins and host inflammatory reaction in response to wounding and infection often lead to extensive tissue damage, with permanent scarring and irreversible loss of vision.[1] The desirable outcome is one in which the immune system can control the infection resulting in reepithelialization and healing with minimum damage to vision. In spite of the significance of the inflammatory response and the immunologic processes occurring in the cornea, the identity of the mediators and their mechanisms of action remain obscure.

The cornea is normally considered a privileged site because of its avascularity and the absence of lymphatic vessels.[8–10] Antigens, cytokines, inflammatory mediators, and leukocytes that enter into the cornea must do so from the limbic and/or ciliary body vessels. Inflammatory cytokines and/or chemotactic gradients that are elicited locally by corneal cells could therefore profoundly affect the emigration of leukocytes from the limbic and ciliary circulation to the cornea. Clearly, the identification of a corneally derived chemotaxin or inflammatory mediator would be of extreme importance in our understanding of the mechanisms that regulate corneal inflammation and healing.

We have identified a novel inflammatory mediator, cationic antimicrobial protein of M_r 37 kDa (CAP37) that plays an important role in host defense and inflammation in the systemic circulation.[11–13] CAP37 is constitutively expressed in the granules of human polymorphonuclear neutrophils (PMNs) and in the α granules of platelets[14,15] and, because of its strong antibiotic activity, is viewed as part of the oxygen-independent killing mechanism of the PMNs.[16–18] The native protein is particularly potent against the Gram-negative organisms *Escherichia coli*, *Salmonella typhimurium*, and *P. aeruginosa*.[16–18] Synthetic peptides based on the native CAP37 sequence have demonstrated antibiotic activity against the Gram-positive organisms *Enterococcus faecalis* and *S. aureus*.[19] In addition to its effects on bacteria, we can now attribute to CAP37 many important functional effects on mammalian cells. We have shown that CAP37 induces powerful chemotactic activity in monocytes[11] and regulates endothelial cell functions, such as stimulating protein kinase C.[20] We can now show that, in addition to the constitutive expression in PMNs, there is a vascular form of CAP37 that can be induced in cerebral microvascular endothelial cells in response to inflammatory mediators such as lipopolysaccharide and cytokines.[21]

To further investigate the biological significance of CAP37 in infection and inflammation, we selected a well-characterized in vivo model of *S. aureus* keratitis,[22,23] to study its contribution to the host defense mechanism. The in vivo studies outlined herein demonstrate the kinetics of expression of CAP37 in extraneutrophilic sites, including corneal epithelium and stromal keratocytes. These findings were further investigated, in in vitro studies in which human corneal epithelial cells (HCECs) and stromal keratocytes were used to determine the mechanism of induction of CAP37 in these cells. Molecular cloning of corneal epithelium–derived CAP37 was undertaken to confirm our immunocytochemical analysis that the corneal

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epithelium-derived protein was unequivocally CAP37. Our findings led us to speculate that CAP37 may have far more wide-ranging effects on the inflammatory process than acting solely as an antibiotic and may indeed play a significant role in the sequential events involved in leukocyte emigration and epithelium–leukocyte interactions in the inflamed cornea after infection.

METHODS

In Vivo Model of *S. aureus* Keratitis

A rabbit model of *S. aureus* keratitis was used to determine the localization of CAP37 in the eye in response to infection. The model is well established and the methodology published previously.[22,23] Maintenance of animals and all in vivo experimentation was performed according to institutional guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Briefly, New Zealand White rabbits (2.0–3.0 kg) were injected intrastromally with approximately 100 colony-forming units of log phase *S. aureus* (RN6390, a wild-type strain generously provided by Ambrose Cheung, Rockefeller University, New York, NY).[24] The contralateral eye was either injected intrastromally with phosphate-buffered saline (PBS, 0.01 M [pH 7.4], containing 0.15 M NaCl; sham control) or was left undisturbed (absolute control). The rabbit eyes were assessed every 5 hours after infection by slit lamp examination (Biomicroscope SL-5D; Topcon, Tokyo, Japan).[22,23,25] The course and severity of *S. aureus* keratitis caused by strain RN6390 in these experiments was found to be similar to that in our previous reports.[22,23,25] Eyes were enucleated at 5, 10, 15, 20, and 25 hours after infection and processed for histologic analysis by fixing in 10% formalin for 24 hours. Embedding, processing, and sectioning of tissue were performed according to standard histologic techniques (Dean McGee Eye Institute, Histology Service Facility, Oklahoma City, OK).

Cell Culture

Immortalized HCECs, kindly provided by Araki-Sasaki (Suita, Japan) were maintained as previously published.[26] Briefly, HCECs were cultured in defined keratinocyte serum-free medium (Gibco BRL, Grand Island, NY) containing 1% penicillin-streptomycin (Gibco BRL). Human stromal keratocytes were derived from donor corneas (North Florida Lions Eye Bank, Jacksonville, FL) and cultured in Dulbecco's modified Eagle's medium (DMEM; Mediatech, Herndon, VA) containing 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT) and 1% penicillin-streptomycin (Gibco BRL), according to our previous methods.[27] Medium changes were made every 2 to 3 days, and cells were subcultured (0.25% trypsin and 1 mM EDTA at 37°C for 5 minutes; Gibco BRL) when they reached 70% confluence at a split ratio of 1:3. For measurement of cell-adhesion molecules, the cells were detached, using 5 mM EDTA alone (37°C for 10 minutes). Cells were transferred to serum-free basic medium overnight before the start of each experiment.

Recombinant CAP37

Functionally active recombinant (r)CAP37 was produced using an RSV-PL4 expression system in human 293 cells.[28,29] The recombinant protein was characterized by amino acid sequence, SDS–polyacrylamide gel electrophoresis, and Western blot analysis and shown to be identical with native PMN-derived CAP37. All preparations of rCAP37 were endotoxin free, as determined by the limulus amebocyte lysate assay (QCL 100l; Whittaker Bioproducts, Walkersville, MD) performed exactly according to the manufacturer's instructions.

Immunohistochemistry

The immunohistochemical analysis performed on paraffin-embedded formalin-fixed rabbit eyes was essentially according to our published methods.[11,21] We used a previously characterized monospecific mouse anti-CAP37 antiserum[11] and the avidin-biotin complex (ABC) technique (Vectastain ABC Elite; Vector Laboratories, Burlingame, CA) to detect CAP37. Briefly, 5-μm sections were cut along the optical axis and, after the various blocking steps,[21] were incubated in the primary antibody (mouse anti-human CAP37 at 1:1000 dilution in PBS containing 0.1% normal goat serum and 0.1% bovine serum albumin) for 60 minutes at room temperature. After three washes in buffer, the slides were incubated for 30 minutes in the secondary antibody (biotinylated goat anti-mouse IgG (Vectastain ABC Elite; Vector Laboratories) and then processed exactly as described in our previous publication.[21] To determine nonspecific staining, negative controls without the primary antibody, normal mouse serum, and immunoadsorbed anti-CAP37 antiserum were incorporated in each experiment. Tissues were viewed under a microscope (BH-2; Olympus, Lake Success, NY) and photographs taken (C-35AD4 camera; Olympus).

For immunocytochemical analysis of HCECs and stromal keratocytes in culture, the cells were cultured on coverslips (Corning Costar, Acton, MA), placed within 24-well tissue culture plates (Corning Costar) until they reached 70% confluence, and immunostained for CAP37, as described earlier, except for the following changes. Cells were fixed in formol-acetone (pH 7.4) for 60 seconds at 4°C[11] and were stained using the mouse anti-human CAP37 antiserum (1:500 dilution).

In Vitro Induction of CAP37 in HCECs and Keratocytes

To determine whether proinflammatory cytokines can induce CAP37 in HCECs and keratocytes, we treated these cell cultures with TNF-α (0–10 ng/mL; Roche Molecular Biochemicals, Indianapolis, IN) and IL-1β (0–20 ng/mL; Endogen, Woburn, MA) for 0 to 24 hours and assayed the cells immunocytochemically for the presence of CAP37 protein, as described earlier. Untreated cell cultures were included for each test sample. In addition to protein detection, upregulation of CAP37 mRNA in response to TNF-α and IL-1β was measured by RT-PCR.

Reverse Transcription–Polymerase Chain Reaction

Cultured HCECs were treated with 5 ng/mL TNF-α and 10 ng/mL IL-1β for 0 to 8 hours at 37°C. Total cellular RNA was isolated from untreated and treated HCECs, according to vendor specifications (TRIzol; Gibco BRL). After reverse transcription of 5 μg total RNA by random oligonucleotide priming (hexanucleotide mix; Roche Molecular Biochemicals, GmbH, Mannheim, Germany), the resultant single-stranded cDNA was amplified by PCR (2400 thermocycler; Perkin Elmer, Foster City, CA) using CAP37 specific primers (5′-CAGAATCAAGGCAGGCACTTCTGC-3′ and 5′-GAGAACACCATCGATCGAGTCTCG-3′) designed for a 597-bp internal fragment of HL60-CAP37.[30] The reaction conditions for reverse transcription were 80 U RNase inhibitor (Sigma, St. Louis, MO), 8 μL 5× strand buffer, 2 μL random hexanucleotide mix, 1 mM dNTPs (Gibco BRL), 10 mM dithiothreitol (Gibco BRL), and 400 U Moloney murine leukemia virus (MMLV) reverse transcriptase (Gibco BRL) in a total volume of 100 μL. The reaction mix was incubated at 37°C for 50 minutes, followed by incubation at 70°C for 15 minutes. The PCR mix (1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1.26 μM of each primer, and 1 U *Taq* polymerase; Gibco BRL) was amplified for 30 cycles. Amplified DNA fragments were separated by electrophoresis on a 1% agarose gel and visualized by exposure to UV after ethidium bromide (0.5 μg/mL) staining. To assess the integrity of the cDNA, primers for human β-actin were used.

Molecular Cloning and Sequencing of HCEC CAP37

The cDNA products from the RT-PCR were excised from the agarose gel, purified (Gene Clean II Kit; Bio 101, Vista, CA), and then cloned (TA Cloning Kit; Invitrogen, Carlsbad, CA) according to the manufac-

turer's instructions. Ten white transformants from each treatment were chosen for plasmid DNA isolation and purification (Wizard Plus SV Miniprep DNA Purification System; Promega, Madison, WI). Plasmids were sequenced (Oklahoma Medical Research Foundation Sequencing Facility and Oklahoma State University Sequencing Facility) in both forward and reverse directions, using the T7 and M13 reverse primers from six different clones from three independent clonings. The resultant sequences were aligned using Pôle Bio-Informatique Lyonnais (PBIL), Network Protein sequence analysis[31] for DNA, and the consensus sequence compared against the HL-60 CAP37 cDNA sequence.[30] (PBIL is a server that provides access to tools for nucleotide and protein sequence analysis, developed by the Laboratory of Biometry and Evolutionary Biology, Villeurbanne, France, and the Institute of Biology and Chemistry of Proteins, Claude Bernard Lyon1 University, Lyon, France; available in the public domain at http://www.pbil.univ.lyon1.fr/pbil.html/.)

Flow Cytometry

Flow cytometry was used to assess the upregulation of ICAM-1 and vascular cell adhesion molecule (VCAM)-1 on HCECs in response to CAP37 treatment. HCECs were cultured as described earlier and treated with CAP37 (0–2000 ng/mL) for 0, 2, 6, 24, 48, and 72 hours. A corresponding culture was left untreated at each time point. After treatment with CAP37, cells were detached with 5 mM EDTA (pH 7.4; Fisher Scientific, Fairlawn, NJ), washed twice in PBS and fixed with 0.125% paraformaldehyde (J. T. Baker, Phillipsburg, NJ) overnight at 4°C. The cells were washed in PBS and incubated in 0.5% normal goat serum and 0.5% BSA in PBS for 30 minutes to block nonspecific binding sites. For determination of ICAM-1 expression, cells were incubated in the primary antibody (FITC-labeled mouse anti-human ICAM-1; Biosource, Camarillo, CA) at 10^6 cells/10 μL at 4°C for 1 hour. Cells were washed in PBS and analyzed by flow cytometry (FACStar; Becton Dickinson, San Jose, CA). For detection of VCAM-1 expression, cells were incubated with unlabeled primary antibody (monoclonal mouse anti human VCAM-1, at 2 μg/10^6 cells; Endogen) followed by FITC-labeled goat anti-mouse IgG (PharMingen, San Diego, CA) at 5 μg/10^6 cells and incubated at 4°C for 30 minutes. The isotype control was FITC-labeled mouse isotype IgG_1 (PharMingen). The positive control was TNF-α (5 ng/mL). Ten thousand cells were analyzed for each sample.

Statistical Analysis

Data from the adhesion molecule studies are presented as mean ± SE. Groups were compared by unpaired Student's *t*-test followed by ANOVA. $P < 0.05$ was considered significant.

Results

In Vivo Expression of CAP37 in an *S. aureus* Keratitis Model

Immunohistochemical analysis was performed on tissue sections obtained from eyes at 5, 10, 15, 20, and 25 hours after injection with *S. aureus*. The initial detection of CAP37 was made in the limbal epithelium and, to a lesser extent, in the corneal epithelium at 5 hours (Fig. 1A). Staining for CAP37 was not observed in sham-injected eyes at 5 hours after infection (Fig. 1B) or at the later time points (not shown). By 10 hours after infection, strong staining for CAP37 was demonstrated in the corneal epithelium (Fig. 1C), ciliary epithelium, related limbus and ciliary vascular endothelium (Fig. 1D), and bulbar conjunctiva, in tissue sections from eyes of rabbits injected with *S. aureus*. Staining for CAP37 was not observed in sections stained with normal mouse serum (Fig. 1E) or with antiserum immunoadsorbed with CAP37 (Fig. 1F). The antibody control in Figures 1E and 1F indicate the specificity of the reaction for CAP37. No PMNs or other leukocytic infiltration was observed in the cornea at the 10-hour time point. However, a few PMNs were seen in the bulbar conjunctiva and the corneal limbus. The strong staining for CAP37 in the corneal epithelium persisted up to 15 hours (Fig. 1G) and began to wane by 20 hours (Fig. 1H). Staining for CAP37 in stromal keratocytes was more marked at the 15-hour time point than at the 10-hour time point. An important observation in this in vivo model was that CAP37 induction in vivo was observed before leukocyte infiltration, which in our studies occurred at 15 hours after infection (Fig. 1G). Neutrophils were first seen in the stroma at approximately 15 hours after injection of the pathogen and then began to accumulate at the base of the epithelial layer between 20 and 25 hours after infection (Fig. 1H). Obvious stromal edema and severe anterior chamber inflammatory reaction were also readily observed at the later time points. With time, the inflammatory reaction became more severe. Clumps of bacteria were evident within the stroma but the levels of CAP37 in the corneal epithelium and stromal keratocytes diminished. It is important to note that PMNs continued to stain for CAP37 throughout all the time points (Fig. 1H), even though epithelial CAP37 was reduced or could no longer be detected.

In Vitro Expression of CAP37 in HCECs and Keratocytes

Because CAP37 was detected in the corneal epithelium and stromal keratocytes in vivo in response to the intrastromal Gram-positive infection but was not present in normal, uninfected eyes, we hypothesized that CAP37 was induced in response to inflammatory mediators and/or cytokines generated as part of the host's defense response to the infection. Two proinflammatory cytokines, TNF-α and IL-1β, are known to be present during the acute stages of a wide range of inflammatory situations[32–35] and have been implicated in gene expression of other chemoattractants, such as IL-8.[36,37] Using immunocytochemistry and RT-PCR, we explored the possibility that they may regulate CAP37 expression in HCECs and keratocytes. The immunocytochemical data presented in Figure 2 demonstrate that CAP37 protein was induced in HCECs in response to TNF-α (Fig. 2A) and IL-1β (Fig. 2B). CAP37 protein was observed as early as 60 minutes in the TNF-α–treated cells and appeared to reach maximum level at 24 hours. Expression of CAP37 in response to IL-1β was observed at a later time point (4 hours) and, similar to TNF-α, appeared to have its maximum effect at 24 hours. There was no constitutive expression of CAP37 protein in untreated HCECs (Fig. 2C). Antibody control using immunoadsorbed anti-CAP37 antiserum showed no staining, indicating the specificity of this reaction (Fig. 2D). Stromal keratocytes treated with TNF-α (Fig. 2E) and IL-1β (not shown) showed the induction of CAP37 protein. Once again, there was no constitutive expression of CAP37 in these cells, as indicated by the absence of staining with the anti-CAP37 antiserum in the untreated cell cultures (not shown). The specificity of this reaction was demonstrated by the absence of staining with the immunoadsorbed antibody control (Fig. 2F).

We corroborated these immunocytochemical data with RT-PCR. HCECs treated with TNF-α (Fig. 3A) and IL-1β (Fig. 3B) showed a time-dependent expression of CAP37 mRNA. Untreated HCECs did not express CAP37 mRNA. However, on treatment with the proinflammatory cytokine TNF-α, HCECs expressed CAP37 mRNA as early as 15 minutes. These levels were maximum between 30 minutes and 2 hours and declined by 4 hours. IL-1β also induced CAP37 mRNA in HCECs. However, the initial expression of CAP37 mRNA was delayed and was not detected until 1 hour after stimulation (Fig. 3B). Furthermore, the effect was more sustained than with TNF-α,

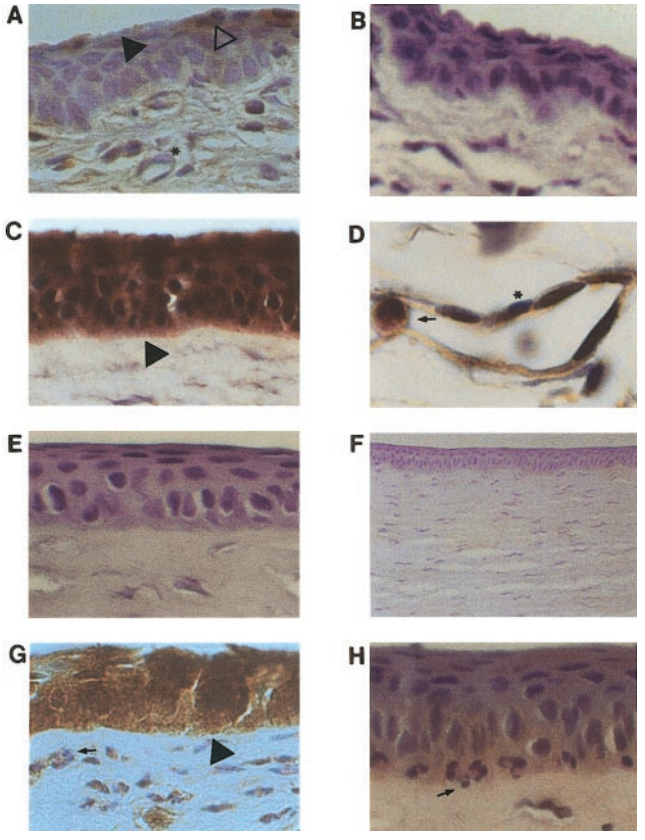

FIGURE 1. Ocular localization of CAP37 in response to intrastromal injection of *S. aureus* in the rabbit eye model of bacterial keratitis. (**A**) A representative photomicrograph of the junction between ocular conjunctiva and cornea at 5 hours after infection. Immunohistochemical staining using mouse anti-CAP37 and the ABC-peroxidase technique indicated faint staining for CAP37 in the conjunctival epithelium (▽), relatively weaker staining in the corneal epithelium (▼), and absence of staining in vascular endothelium (✲). (**B**) Sham-injected rabbit eye at 5 hours stained with mouse anti-CAP37 antiserum shows absence of staining for CAP37 in ocular tissue. (**C**) Immunohistochemical staining for CAP37 with mouse anti-CAP37 antiserum at 10 hours after infection shows strong reaction for CAP37 in corneal epithelium (▼). (**D**) Strong staining for CAP37 in endothelial cells lining a vessel located in the ciliary body at 10 hours after infection (✲). Also note staining for PMN (←). (**E**) Specificity control using normal mouse serum to stain tissue from a rabbit 10 hours after infection. (**F**) Absence of staining with immunoadsorbed anti-CAP37 antiserum in corneal epithelium obtained from rabbit 10 hours after infection. (**G**) Immunohistochemical localization of CAP37 in rabbit eye 15 hours after infection. Strong positive reaction for CAP37 is observed in corneal epithelium (▼) as well as in infiltrating PMNs (←) in corneal stroma and stromal keratocytes. (**H**) Immunohistochemical localization of CAP37 20 hours after infection, indicating reduced levels of staining for CAP37 in corneal epithelium. Note continued strong staining for CAP37 in PMN (←) at the base of the epithelial layer. Magnification, (**A–C**, **E**, **G**, **H**) ×400; (**D**) ×1000; (**F**) ×100.

because the message could be detected even at 6 hours. These findings corroborate our immunocytochemical data showing that TNF-α induced protein at an earlier time point and that IL-1β elicited the more intense staining of CAP37.

Molecular Cloning of Human Corneal Epithelial Cell CAP37

To determine whether epithelial CAP37 was similar to PMN-derived CAP37 we undertook the cloning of HCEC CAP37. Total cellular RNA was isolated from HCECs treated with TNF-α for 2 hours and cDNA synthesis performed according to the method described earlier. RT-PCR was used to amplify the CAP37 gene from HCECs using the pair of oligonucleotide primers, as described in the Methods section, and based on a previously published cDNA sequence of CAP37.[30] Comparison of the PMN and epithelial–CAP37 sequences between amino acids 20 and 218 showed a complete match except for amino acid residue 132, where a histidine residue (epithelial CAP37) consistently replaced the arginine residue normally found in PMN-CAP37 (Fig. 4).

Upregulation of ICAM-1 and VCAM-1 on Cultured HCECs

Because our in vivo data demonstrated the adherence of large numbers of PMNs to corneal epithelial cells, we speculated that this was due to the upregulation of cell adhesion molecules on the epithelial cells. In vitro studies were undertaken to investigate the effect of CAP37 on the upregulation of ICAM-1 on HCECs. Cells were treated with CAP37 (0–2000 ng/mL) for 0 to 72 hours, and levels of ICAM-1 were measured by flow cytometry. ICAM-1 was upregulated by CAP37 in a dose-dependent manner, with maximum upregulation obtained with 1000 to 2000 ng/mL CAP37 (Fig. 5A). These levels were comparable to those obtained with the positive control TNF-α (5 ng/mL). Lower, yet significant, levels of ICAM-1 were obtained with CAP37 at concentrations between 10 and 500 ng/mL. Kinetic

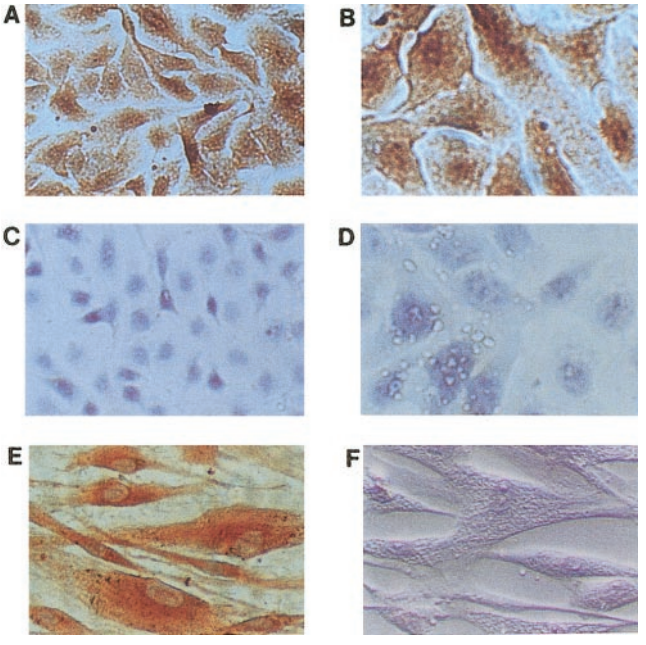

FIGURE 2. In vitro induction of CAP37 in HCECs and stromal keratocytes. (**A**) Representative micrographs showing immunohistochemical detection of CAP37 protein with mouse anti-CAP37 antiserum and the ABC-peroxidase staining technique on HCECs treated with TNF-α (5 ng/mL) for 24 hours. (**B**) Induction of CAP37 in HCECs in response to IL-1β (10 ng/mL) treatment for 24 hours. (**C**) Staining using mouse anti-CAP37 antiserum on untreated HCECs indicating absence of staining for CAP37. (**D**) Antibody control using immunoadsorbed anti-CAP37 antiserum on HCECs treated with IL-1β (10 ng/mL) for 24 hours. (**E**) Induction of CAP37 in stromal keratocytes in response to TNF-α (10 ng/mL for 24 hours) as detected immunohistochemically with mouse-anti-CAP37 antiserum. (**F**) Immunoadsorbed anti-CAP37 antiserum control indicating absence of CAP37 in stromal keratocytes treated with TNF-α (10 ng/mL for 24 hours). Magnification, (**A**, **D**) ×200; (**B**, **E**, **F**) ×400; (**C**) ×100.

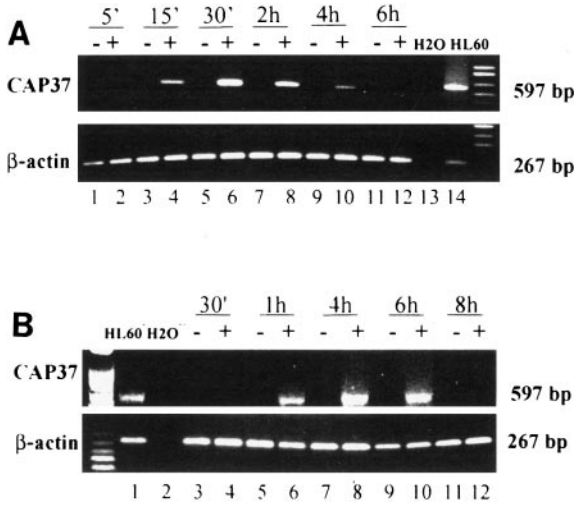

FIGURE 3. Kinetic study demonstrating the effect of proinflammatory cytokines on steady state levels of CAP37 mRNA in corneal epithelial cells. (**A**) HCECs were treated (+) with TNF-α (5 ng/mL) for 5, 15, and 30 minutes and 2, 4, and 6 hours, and CAP37 mRNA expression (*top*, 597 bp) was determined by RT-PCR (*lanes 1–12*). Untreated (−) HCEC control cultures are included at each time point. *Lane 13*: negative water control; *lane 14*: the positive HL-60 control. (**B**) HCECs were treated with IL-1β (10 ng/mL) for 0.5, 1, 4, 6, and 8 hours and CAP37 mRNA expression (*top*, 597 bp) determined by RT-PCR (*lanes 3–12*). Untreated (−) controls are included for each incubation point. *Lane 1*: the positive HL-60 control; *lane 2*: negative water control. (**A**, **B**, *bottom*) cDNA integrity as assessed with the β-actin primer (267 bp). Molecular markers are shown in unmarked lanes in all panels.

studies (Fig. 5B) indicated that HCECs did not constitutively express ICAM-1 and that no upregulation of ICAM-1 could be detected by flow cytometry at the early time point of 2 hours. However, by 6 hours, significant upregulation of ICAM-1 was observed. The levels declined by 24 hours but were still above the untreated levels. CAP37 failed to upregulate VCAM-1 on HCECs as determined by flow cytometry as well as RT-PCR (not shown).

DISCUSSION

Extravasation of leukocytes from the circulation into tissue sites is an integral feature of the host response to injury and inflammation.[38] By virtue of their ability to engulf and destroy bacteria, eliminate toxins, and secrete numerous soluble mediators, leukocytes are capable of restricting and limiting the spread of infection. In the acute stages of most infections, the predominant cell type is the PMN.[38,39] This observation held true in our in vivo rabbit model of *S. aureus* keratitis, in which the primary leukocyte observed in the initial 25-hour period after infection was the PMN. The rabbit bacterial keratitis model indicated the expected expression of CAP37 in the granules of migrating PMNs. However, an unexpected observation was the expression of CAP37 in corneal epithelial cells, stromal keratocytes, ciliary epithelium, related limbus and ciliary vascular endothelium, and bulbar conjunctiva. Particularly striking was the extremely strong staining for CAP37 in corneal epithelium at 10 hours after infection. The induction of CAP37 in the cornea occurred before the migration of PMNs, which in this model occurred approximately 15 hours after infection. The path of migration of PMNs appeared to be from ciliary and limbal vessels through the stroma to the basal aspects of the epithelial layer, where large numbers of PMNs were seen to accumulate.

Clearly, extraneutrophilic CAP37 is induced in response to infection or an inflammatory stimulus, because sham-injected animals did not show staining for CAP37. These are significant findings, because the extraneutrophilic localization of CAP37 in ocular tissue in response to infection has not been reported previously. Our data strongly suggest that the source of CAP37 in the corneal epithelium is endogenous during the early stages of infection. This is based on our unequivocal observations that corneal CAP37 was seen in the absence of and before PMN extravasation. Thus, the staining observed in the epithelium could not be due to exogenously released CAP37 from PMNs. However, later in the infection, once PMNs migrate to the cornea, the possibility certainly exists that the staining observed is a combination of both exogenous and endogenous CAP37. The question of whether the induction of CAP37 is specific to infection caused by a Gram-positive organism or

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PMN-CAP37   20  NQGRHFCGGA LIHARFVMTA ASCFQSQNPG VSTVVLGAYD LRRRERQSRQ  69
                |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
EPI-CAP         NQGRHFCGGA LIHARFVMTA ASCFQSQNPG VSTVVLGAYD LRRRERQSRQ
                ---------- Antibiotic ----------

PMN-CAP37   70  TFSISSMSEN GYDPQQNLND LMLLQLDREA NLTSSVTILP LPLQNATVEA  119
                |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
EPI-CAP         TFSISSMSEN GYDPQQNLND LMLLQLDREA NLTSSVTILP LPLQNATVEA
                                                ---------- PKC1 ----------
                                                ----------

PMN-CAP37   120 GTRCQVAGWG SQRSGGRLSR FPRFVNVTVT PEDQCRPNNV CTGVLTRRGG  169
                |||||||||| || ||||||| |||||||||| |||||||||| ||||||||||
EPI-CAP         GTRCQVAGWG SQHSGGRLSR FPRFVNVTVT PEDQCRPNNV CTGVLTRRGG
                ---------- ?Chemotactic ----------  ---------- PKC2 ----------

PMN-CAP37   170 ICNGDGGTPL VCEGLAHBVA SFSLGPCGRG PDFFTRVALF RDWIDGVLN   218
                |||||||||| |||||||||| |||||||||| |||||||||| |||||||||
EPI-CAP         ICNGDGGTPL VCEGLAHBVA SFSLGPCGRG PDFFTRVALF RDWIDGVLN
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FIGURE 4. Alignment of human corneal epithelial cell–derived CAP37 with a human neutrophil-derived CAP37 sequence. The known sequence of PMN-CAP37 compared with the deduced amino acid sequence of HCEC-CAP37 between amino acid residues 20 and 218. Amino acid residue Arg132 is replaced by a His residue in the HCEC-CAP37 sequence (*bold type*). *Underscored* peptide 20 to 44 (Antibiotic) denotes antibiotic domain[19]; peptide 95 to 122 (PKC_1) denotes the primary PKC-activating domain; peptide 140 to 165 (PKC_2) denotes a secondary domain with PKC activity[20]; peptide 95 to 165 (?Chemotactic) denotes putative chemotactic region.

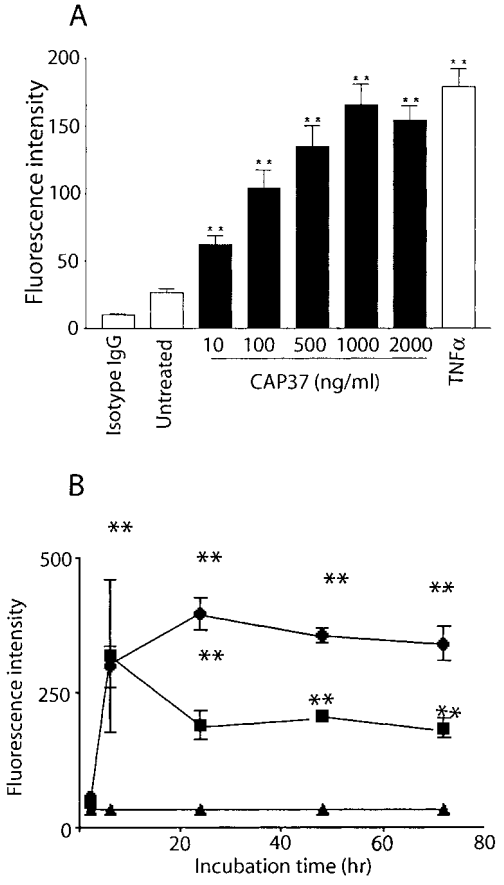

FIGURE 5. Upregulation of ICAM-1 on HCECs in response to CAP37. (**A**) Dose–response effect of CAP37 on expression of ICAM-1 on cultured HCECs. Cells were treated with CAP37 (0–2000 ng/mL) for 6 hours and stained with FITC-labeled mouse anti-human ICAM-1, and fluorescence intensity was measured by flow cytometry. Controls included the isotype IgG_1 antibody and TNF-α (5 ng/mL), which served as the positive control. Data are the mean ± SE of results obtained from nine independent experiments. (**B**) Kinetic response of CAP37-mediated expression of ICAM-1 in HCECs. Cells were treated with 1000 ng/mL of CAP37 (●) at 2, 6, 24, 48, and 72 hours, and ICAM-1 expression was analyzed by flow cytometry. TNF-α at 5 ng/mL (■) was used as the positive control. (▲) Untreated control. Values are the mean ± SE of results obtained from three independent experiments. $^{**}P < 0.01$ compared with untreated control.

whether its expression can also be upregulated as a result of Gram-negative and viral infections cannot be answered in this study. Our in vitro studies depicted in Figures 2 and 3 support the concept that CAP37 can be induced in any ocular infection in which TNF-α and IL-1β are generated. However, definitive studies are currently in progress to examine the expression of CAP37 in an in vivo model of bacterial keratitis using the Gram-negative organism *P. aeruginosa* to ascertain the specificity of this induction.

In our in vitro studies, the proinflammatory mediators TNF-α and IL-1β regulated CAP37 expression in corneal epithelial cells and stromal keratocytes in a time- and dose-dependent fashion. Untreated cells did not display CAP37 message or protein, indicating that it was not constitutively expressed in either of these cells. To our knowledge, this is the first demonstration of the expression of a monocyte chemoattractant in HCECs in response to cytokines. The induction of monocyte chemotactic protein (MCP)-1, regulated on activation normal T-cell expressed and secreted (RANTES),[40] and GRO-α,[41] members of the C-C chemokine family with chemotactic effects on monocytes, has been demonstrated in stromal keratocytes, but not in HCECs. In contrast, expression of C-X-C chemoattractants, such as IL-8 with potent effect on PMN migration, can be induced in HCECs[36] and stromal keratocytes.[27,37] The potency of CAP37 in in vivo recruitment of leukocytes in ocular infections in comparison to other chemoattractants, including macrophage inflammatory protein (MIP)-1α, MIP-2, and KC,[42–45] could not be assessed in these studies. This in no way diminishes the relevance of our findings but can only stress the importance of these first studies that demonstrate a novel localization of the inflammatory mediator CAP37 and suggest that these new properties contribute to its role in host defense in ocular inflammation in ways yet to be determined.

Previous studies on CAP37 have shown unequivocally that it has no effect on PMN migration[11] or activation (Pereira HA, Kumar P, unpublished observations, 1999). The findings reported herein clearly pose at least two intriguing questions. First, if the role of CAP37 in the corneal epithelium is to serve as a chemoattractant, why did we not observe monocyte emigration during the time course of CAP37 expression in the cornea? Secondly, why did PMNs rather than monocytes accumulate and adhere to the corneal epithelial layer in response to the expression of CAP37? A simple answer to the first question is that the time course of our in vivo experiments was too short to observe monocyte migration. Another more complex interpretation may be derived from our sequence data. We have previously defined the antibiotic domain of the CAP37 molecule to reside between amino acid residues 20 and 44,[19,46] and the corneal epithelium-derived sequence would imply that the antimicrobial activity is retained. We have yet to elucidate the chemotactic domain, but our ongoing studies suggest that it lies between amino acids 95 and 165. It is intriguing to speculate that the replacement of the arginine residue by a histidine residue at position 132 could be sufficiently critical to contribute to a loss of chemotactic activity. Studies are planned to investigate this hypothesis by synthesizing peptides based on the epithelial CAP37 sequence and comparing it with PMN-derived peptides in an in vitro chemotaxis assay. Ongoing experimentation to establish the complete amino acid sequence of epithelial CAP37 will provide evidence regarding further variations from the PMN-CAP37 sequence and help evaluate the structure–function relationships of this molecule.

To further explore the controversial findings that PMNs and not monocytes adhered strongly to corneal epithelial cells, we undertook a series of studies in which the effect of CAP37 on the upregulation of adhesion molecules ICAM-1 and VCAM-1 on the surface of HCECs was investigated. ICAM-1, a member of the immunoglobulin superfamily class of adhesion molecules has been identified on corneal epithelium.[47–49] Its upregulation on corneal epithelial cells promotes adherence of PMNs and monocytes through its interactions with the leukocyte function antigen (LFA)-1 receptor (CD11a/CD18) present on the surface of all leukocytes[50–52] and Mac-1 (CD11b/CD18), which is present on PMNs and monocytes.[53] The adherence of monocytes is further facilitated by VCAM-1, through the ligand VLA-4, which is present on monocytes and lymphocytes but not on PMNs.[54] The specificity of our findings for PMN–epithelium interactions rather than monocyte–epithelium interactions may be explained by our key findings that VCAM-1 is not upregulated on these cells in response to CAP37. That there was no upregulation of VCAM-1 by CAP37 on HCECs is not entirely surprising, because although it is inducible by cytokines on endothelial cells and on certain subsets of nonvascular cells, including thymic, airway, and gut mucosal epithelial cells, it has not been identified on corneal epithelial cells to date.[48]

It is becoming increasingly apparent that the role of CAP37 in infection is multifunctional. This is exemplified in our cur-

rent model of bacterial keratitis. The induction of CAP37 in the cornea in response to infection strongly supports our hypothesis of its role in the innate defense mechanism of the host. It stands to reason that an antibacterial peptide that is located in the outermost layers of the eye and that can be induced during the very early phases of an infection would be of great benefit to the host. Epithelial expression of CAP37 would thus serve as an important mediator in the first line of defense, even before the arrival of PMNs. Furthermore, the induction of corneal epithelial CAP37 may have an autocrine effect on epithelial cells or a paracrine effect on neighboring cells. Once PMNs arrive at the site of infection, PMN-derived CAP37 could regulate epithelial cell functions in an exocrine fashion. Although, we are still uncertain whether epithelial CAP37 has monocyte–chemotaxis activity, PMN-derived CAP37 could set up a chemotactic gradient serving to bring monocytes in from the limbic circulation. We speculate that CAP37 plays a pivotal role in the inflammatory response of the cornea to infection through its ability to act as an antibiotic, elicit leukocyte emigration, modulate corneal epithelial adhesion molecules, and promote leukocyte-epithelium interactions.

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