Expression and Regulation of Antimicrobial Peptide Psoriasin (S100A7) at the Ocular Surface and in the Lacrimal Apparatus

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PURPOSE. Psoriasin, originally isolated from psoriasis as an overexpressed molecule of unknown function, has recently been identified as a principal Escherichia coli-killing antimicrobial peptide of healthy skin. The purpose of this study was to investigate the expression and antimicrobial role of psoriasin at the ocular surface and in the lacrimal apparatus.

METHODS. Different tissues of the lacrimal apparatus and ocular surface were systematically analyzed by means of RT-PCR, Western blot, and immunohistochemistry for their ability to express and produce psoriasin. The inducibility and regulation of psoriasin were studied in human corneal as well as conjunctival epithelial cell lines after challenge with ocular pathogens and proinflammatory cytokines. Real-time RT-PCR was performed to evaluate the expression and induction of psoriasin. In addition, tear fluid obtained from different healthy volunteers was examined by ELISA for its psoriasin concentration.

RESULTS. RT-PCR and Western blot analyses revealed a constitutive expression of psoriasin in cornea, conjunctiva, nasolacrimal ducts, and lacrimal gland. Immunohistochemistry showed strong staining of meibomian glands for psoriasin. No induction of psoriasin was observed after stimulation with supernatants of E. coli, whereas supernatants of Staphylococcus aureus and Haemophilus influenzae significantly increased the psoriasin mRNA expression. Stimulation with IL-1β and VEGF also strongly increased psoriasin transcription. The highest amounts of psoriasin protein were detected in the tear fluid (~170 ng/mL) of healthy volunteers.

CONCLUSIONS. The results suggest that psoriasin is produced by the structures of the ocular surface and is part of the innate immune system at the ocular surface and tear film. (Invest Ophthalmol Vis Sci. 2011;52:4914 – 4922) DOI:10.1167/iovs.10-46598

As a mucosa, the ocular surface is in constant interaction with the environment and thus also with diverse bacteria, bacterial components, and their pathogen-associated molecular patterns (PAMPs). The continuous lacrimation with tear fluid and the wiper function of the eyelids are mechanically effective characteristics that counteract colonization and invasion of microorganism at the ocular surface. In addition, the tear film contains a bulk of unspecific, antibacterial proteins, such as lysozyme, lactoferrin, lipocalins, secretory phospholipase A2, and components of the complement system.1,2 Another primary defense mechanism of the innate immune system at the ocular surface is antimicrobial peptides (AMPs). These small, cationic peptides demonstrate an antimicrobial activity against a broad spectrum of bacteria, fungi, and viruses. Furthermore, several AMPs modulate cellular activation processes like migration, proliferation, chemotaxis, and cytokine production and so also affect the adaptive immune system.3,4 The major AMPs detected at the ocular surface so far are human cathelicidin LL-37 and defensins (α- and β-defensins).3,4 In addition, recent studies describe several additional AMPs that are produced by the ocular surface epithelia, such as liver-expressed antimicrobial peptides (LEAP)-1 and -2,5 macrophage inflammatory protein (MIP)-3α,7 DEFB-109,15,16 and RNase7,17

During recent years, the protein psoriasin (S100A7c) has been characterized as an additional AMP that protects human skin from Escherichia coli.18,19 Psoriasin is a member of the S100 multigene family that is encoded in the epidermal differentiation complex (EDC) which is located on chromosome 1, region q21. This chromosomal region encodes a multiplicity of genes that are involved in the formation of the cornified cell envelope (CE) of the human epidermis.19,20 S100 proteins are characterized by two calcium-binding EF hands (helix–loop–helix domains) and low molecular weight. Because of their function as calcium sensors, S100 proteins regulate the function and/or subcellular distribution of specific target proteins.21 Psoriasin was first isolated and identified from psoriatic keratinocytes22 and is constitutively produced in lower concentration in normal healthy epidermis.18,19 Primary characterization as a marker of psoriasis, psoriasin expression is induced in many epithelial inflammatory diseases (e.g., atopic dermatitis) and invasive cancer.24 Gläser et al.19 have identified psoriasin as an E. coli-killing antimicrobial peptide of healthy skin. At higher concentrations, it exhibits bactericidal activity against Gram-negative Pseudomonas aeruginosa and Gram-positive Staphylococcus aureus.19,25 Sequestration of trace elements such as zinc and the direct permeabilization of bacterial membranes at lower pH have been proposed as the killing mecha-
nsms. Furthermore, recent studies have indicated that psoriasin expression is upregulated by proinflammatory cytokines, bacterial compounds,9,25 UV-B radiation,26 and rising intracellular calcium concentration29 in human epithelium and/or cultured keratinocytes. The upregulation of psoriasin is triggered by the bacterial component flagellin via Toll-like receptor (TLR) 5, the specific flagellin ligand.30 It has also been shown to be a potent and selective chemotactic agent for CD4+ T lymphocytes and neutrophils at sites of inflammation or infection.31,32 RAGE, the receptor for advanced glycation end products, has been shown to mediate the chemotactic function of psoriasin.33

In the present study, we tested the hypothesis of whether human psoriasin is also part of the innate defense system of the ocular surface, tissues of the lacrimal apparatus, and the tear film and whether it is inducible at the ocular surface.

**Materials and Methods**

**Tissues and Cell Lines**

The study conformed with Institutional Review Board regulations, informed consent regulations, and the provisions of the Declaration of Helsinki.

Tissues of the ocular surface and the lacrimal apparatus were obtained from cadavers (nine male, eleven female, aged 41–93 years) donated to the Department of Anatomy and Cell Biology, Martin Luther University Halle-Wittenberg, Germany. The donors were free of recent trauma, eye and nasal infections, and diseases involving or affecting lacrimal function. All the tissues were dissected from the cadavers within a time frame of 4 to 24 hours postmortem. After dissection, the tissues were prepared for paraffin embedding (right eye) by 4% paraformaldehyde fixation or were used for molecular biological investigations (left eye) and were immediately frozen at −80°C.

SV40-transformed human corneal epithelial (HCE) cells (a kind gift from Kaoru Araki-Sasai, Tane Memorial Eye Hospital, Osaka, Japan),34 as well as a human spontaneously immortalized epithelial cell line from normal human conjunctiva (IOBA-NHC, here referred to as HCjE cells; a kind gift from Yolanda Dziebold, University Institute of Ophthalmomibiology [IOBA], University of Valladolid, Valladolid, Spain)35 were cultured as monolayers and used for stimulation experiments.

**Cell Culture**

HCE cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM/HAMS F12 1:1; PAA Laboratories GmbH, Pasching, Austria) containing 10% fetal calf serum (FCS; Biochrom AG, Berlin, Germany), and HCjE cells were cultured in DMEM/HAMS F12 1:1 containing 10% FCS (Biochrom AG), insulin (1 µg/mL; Sigma-Aldrich, Steinheim, Germany), and hydrocortisone (5 µg/mL; Sigma-Aldrich) in a humidified 5% CO2 incubator at 37°C. For stimulation experiments, the HCE and HCjE cells (5 × 105) were seeded in Petri dishes and incubated. At confluence and before treatment, the cells were washed in phosphate-buffered saline (PBS) and incubated in serum-free medium overnight. They were then treated with different dilutions of supernatants of bacteria, the cells were incubated with trypsin soy broth (TSB) medium as a control or in the case of proinflammatory mediators, were stimulated with the solvent. All experimental procedures were performed under normoxic conditions. On completion of each experiment cells and supernatants were collected and stored at −80°C until they were processed for RNA extraction (cells) or analysis of psoriasin secretion (culture supernatants) by ELISA experiments.

**Production of Bacterial Supernatants**

Laboratory strains of S. aureus SA113 (ATCC 35,556), E. coli (ATCC 8759), and H. influenza (Hi) isolates from patients with respective ocular surface inflammation and corneal ulceration were grown overnight at 37°C by shaking in tryptone soy broth (Oxoid, Basingstoke, UK). Thereafter, 10-fold bacterial dilutions were plated on Columbia agar supplemented with 10% sheep blood (Heipha, Eppelheim, Germany), incubated overnight at 37°C, and plate counts were performed. Bacteria were centrifuged twice at 6000 rpm for 30 minutes. Supernatants were filtered twice using filters impermeable to bacteria (0.2-µm pore size; Millipore, Eschborn, Germany). Aliquots of the supernatants were proven to be sterile by overnight incubation on agar. Supernatants were adjusted to a bacterial concentration of 5 × 107 cfu/mL. Supernatants with TSB growth medium (diluted 1:100) were used to analyze non-specific effects of the bacterial supernatants.

**RNA Preparation and cDNA Synthesis**

For conventional reverse transcriptase polymerase chain reaction (RT-PCR), tissue biopsies of the ocular surface (cornea and conjunctiva, n = 20, respectively) and the lacrimal apparatus (lacrimal gland and nasolacrimal ducts, n = 20, respectively) were crushed in an agate mortar under liquid nitrogen, then homogenized (Polytron, Norcross, GA). Total RNA was extracted from the tissue biopsies by (RNeasy Mini Kit; Qiagen, Hilden, Germany). Total RNA was extracted from cultured HCE and HCjE cells (Tetizol reagent; Invitrogen, Karlsruhe, Germany). Crude RNA was purified with isopropanol and repeated ethanol precipitation, and contaminated DNA was destroyed by digestion with RNase-free DNase I (30 minutes 37°C; Boehringer, Mannheim, Germany). DNAse was heat-inactivated for 10 minutes at 65°C. Reverse transcription of all RNA samples to first-strand cDNA (RevertAid H Minus M-Mul; V Reverse Transcriptase Kit; Fermentas, St. Leon-Rot, Germany) was performed according to the manufacturer’s protocol. Two micrograms total RNA and 10 pmol oligo (dT)18 primer (Fermentas) were used for each reaction. The ubiquitously expressed β-actin which proved amplifiable in each case with the specific primer pair, served as the internal control for the integrity of the translated cDNA.

**Polymerase Chain Reaction**

For conventional PCR, each reaction was prepared with 1 µL cDNA (from each sample), 25.72 µL H2O, 0.9 µL 50 mM MgCl2, 0.5 µL 10 mM dNTPs, 3 µL 10× PCR buffer, 0.18 µL (1.25 U) Taq-Polymerase (Invitrogen), and 0.6 µL 10 pmol psoriasin primer mix. The PCR reaction included an initial cycle at 95°C for 2 minutes followed by 35 cycles of 95°C for 20 seconds, 62°C for 30 seconds, 72°C for 30 seconds, and a final elongation at 72°C for 5 minutes with a temperature hold at 4°C. The psoriasis primers used were as follows: forward 5′-CACAGATCT- CAGTGTCTCTTACGTCG-3′ and reverse 5′-GTCTCTTCTGTTGAT- CAGAG-3′ (GenBank accession number NM_002963.3; http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD). The primers were synthesized at MWG-Biotech AG (Ebersberg, Germany).

For PCR control samples (labeled as Ø; RevertAid H Minus M-Mul; V reverse transcriptase was replaced by RNase- and DNase-free water during cDNA synthesis. Ten microliters of the PCR were loaded on a 1.5% agarose gel, and the amplified products were visualized via fluorescence after electrophoresis. Base pair (bp) values were compared with GenBank data. PCR products were also confirmed by sequencing (BigDye; Applied Biosystems, Inc. [ABI]; Foster City, CA).

**Quantitative Real-Time RT-PCR**

Samples were analyzed by real-time RT-PCR (Opticon 2 System; MJ Research, Waltham, MA). Real-time RT-PCR was performed with ps-
Psoriasin released in the cell-free supernatant from nonstimulated or stimulated HCE or HCjE cells, as well as psoriasin concentrations in lacrimal gland, nasolacrimal ducts, conjunctivae, and cornea (n = 20 for each tissue). RT-PCR was performed in triplicate, and the changes in gene expression were calculated by the ΔΔCt method. To confirm the amplification, the resulting realtime RT-PCR products were visualized in an agarose gel.

Immunohistochemistry

For analysis by immunohistochemistry, cadaveric lacrimal glands, upper eye lids with conjunctiva, corneae, and nasolacrimal ducts were fixed in 4% formalin, embedded in paraffin, sectioned (7 μm), and dewaxed. Immunohistochemical staining was performed with a monoclonal mouse antibody to human psoriasin (IMG-409A; Imgenex, San Diego, CA). Sections were microwaved for 10 minutes in 10 mM citrate buffer (pH 6.0) containing 0.1 mol citric acid and 0.1 mol sodium citrate in dH2O, and nonspecific binding was inhibited by incubation with secondary antibody–specific normal serum (Dako) 1:5 in Tris-buffered saline (TBS). The primary antibody (goat anti-mouse IgG; sc-2005; Santa Cruz, Heidelberg, Germany) was incubated at room temperature for at least 2 hours. Visualization was achieved with horseradish peroxidase–labeled streptavidin–biotin complex (StreptABComplex/HRP; Dako) and 3-amin-9-ethylcarbazole (AEC; Dako) for at least 5 minutes. After the sections were counterstained with hemalum, they were mounted (Aquatex; Boehringer, Mannheim, Germany). Furthermore, control sections were incubated with nonimmune IgG to determine possible nonspecific binding of mouse IgG. Positive control sections included human skin or eyelid sections with human skin (obtained from some of the cadavers used in this study). The slides were examined by microscope (Axiophot; Carl Zeiss Meditec, Oberkochen, Germany).

Enzyme-Linked Immunosorbent Assay

Psoriasin released in the cell-free supernatant from nonstimulated or stimulated HCE or HCjE cells, as well as psoriasin concentrations in samples from human cadaveric conjunctivae (n = 4), lacrimal glands (n = 7), and nasolacrimal ducts (n = 4) and reflex tears of four healthy volunteers were measured with an enzyme-linked immunosorbent assay (ELISA), as recently described in detail. The detection limit for the psoriasin ELISA was 0.3 ng/mL. Standard, sample supernatants and negative controls were performed in duplicate.

Western Blot

For Western blot analysis, tissue samples of the ocular surface (cornea and conjunctiva, n = 4, respectively) and the lacrimal apparatus (upper eye lids with cadaveric meibomian glands, lacrimal glands, and nasolacrimal ducts; n = 4, respectively) were crushed in an agate mortar under liquid nitrogen, then homogenized in 300 μL 1% Triton buffer (Triton X-100; Carl Roth, Karlsruhe, Germany) with a protease and phosphatase inhibitor cocktail (Fermentas). The samples were centrifuged at 13,000 rpm for 30 minutes, and the supernatant was stored at −80°C until use. The total protein content was measured with a protein assay (Bio-Rad, München, Germany). Total protein (20 μg) of each tissue was heated at 90°C for 5 minutes in 15 μL RSB buffer and electrophoresed on 12% SDS-PAGE. Proteins were electrotransferred to nitrocellulose membranes. The membranes were blocked for 1 hour in Tris-buffered saline containing 0.1% Triton (TBST) with 5% BSA at room temperature. The blots were incubated in TBST containing 5% BSA with monoclonal mouse antibody to human psoriasin (IMG-409A; Imgenex) at a dilution of 1:500 overnight at 4°C and incubated for 2 hours with goat anti-mouse IgG (1:5000; DakoCytomation, Zug, Switzerland) in 5% nonfat milk powder/TBST at room temperature. Afterward, immunoreactive signals were visualized by chemiluminescence detection (ECL Plus; Amersham Biosciences, Freiburg, Germany). To ensure equal amounts of protein, we used a human β-actin antibody (1:40,000; A5441; Sigma-Aldrich) as a loading control. The molecular weights of the detected protein bands were determined by comparison with standard molecular weight markers (Prestained Protein Ladder; Fermentas).

Statistical Analysis

Data are expressed as the mean ± SE (SEM) of tested samples. Statistical significance was evaluated with the Student’s t-test (InStat statistical software; GraphPad Software, San Diego, CA). P < 0.05 indicated significance.

Results

Expression of Psoriasin mRNA and Protein in Tissues of the Ocular Surface and Lacrimal Apparatus and in Tear Fluid of Healthy Donors

Expression of psoriasin in cadaveric tissues of the ocular surface and lacrimal apparatus was monitored by RT-PCR. Psoriasin-specific cDNA amplification products (178 bp) were detected in lacrimal gland, nasolacrimal ducts, conjunctivae, and cornea (n = 20 for each tissue; Fig. 1) by means of RT-PCR. Psoriasin PCR fragments were also detected in cultivated HCE and HCjE cells. The β-actin control PCR (275 bp) was positive and of similar amounts for all investigated tissues. Negative signals in the PCR control reactions (Ø) performed without reverse transcriptase during cDNA synthesis confirmed specific amplification of only cDNA and excluded primer binding to genomic DNA. Base pair values were equivalent to the expected DNA products compared with GenBank data, and PCR products were also confirmed by sequencing and sequence alignment (data not shown). Five samples each of human cornea, conjunctiva, lacrimal gland, and nasolacrimal ducts were dissected from cadavers (n = 5). Extracts from these samples were tested for psoriasin protein by Western blot analysis. Proteins were separated by SDS-PAGE under reducing conditions and showed distinct bands for psoriasin...
revealed intracytoplasmic reactivity in several cells within the epithelial layer, but not in the secretory product of goblet cells and basal cells, revealing subjectively strong positive intracellular reactivity with psoriasin antibody. Psoriasin expression was detected intracytoplasmically in high columnar epithelial cells and basal cells of nasolacrimal ducts. Seven samples revealed no reaction (not significant). Psoriasin expression is demonstrated in Figure 4.

For the investigation of the distribution of psoriasin in cadaveric, paraffin-embedded tissue, 7-μm sections from upper and lower eyelids (including meibomian glands), cornea, conjunctivae, and nasolacrimal ducts were analyzed. Control sections (nonimmune IgG) were negative (unstained) for each of the investigated tissues. Tissue sections used for positive control indicated that the method applied worked well (not shown). Psoriasin immunoreactivity was detected weakly in corneal epithelium, slightly higher in conjunctival epithelium, lacrimal gland, and nasolacrimal ducts and strongly in the epidermis, meibomian gland, and hair follicles of the eye lid. All ocular samples, except human lacrimal glands (11 positive, 7 negative), exhibited the same staining pattern. Examples of the localization of psoriasin expression are demonstrated in Figure 4. In the cornea, all sections showed only a weak immunoreactivity. Only superficial corneal epithelial cells revealed a weak expression of psoriasin, whereas all other corneal layers showed no reactivity. In the conjunctiva, all sections revealed positive intracellular reactivity with psoriasin antibody. Psoriasin was localized intracytoplasmically in high columnar epithelial cells as well as basal cells, revealing subjectively strong staining near the epithelial surface. The secretory products of goblet cells and goblet cells alone demonstrated no reactivity. In the lacrimal gland, 11 of 18 cases demonstrated positive reactivity. Psoriasin expression was detected intracytoplasmically in acinus cells. Seven samples revealed no reaction (not shown); there was no dependency on age and sex. In the nasolacrimal ducts, positive reactivity was detected intracytoplasmically in high columnar epithelial cells and basal cells of the epithelial layer, but not in the secretory product of goblet cells. Moreover, staining with antibodies against psoriasin also revealed intracytoplasmic reactivity in several cells within the lamina propria beneath the epithelium. In the eyelid, positive reactivity for psoriasin was clearly visible in several structures of all sections analyzed. The squamous epithelium showed positive immunoreactivity in all layers, strong reactivity was visible in the stratum granulosum and stratum corneum. Also, strong immunoreactivity was visible in the hair shaft and around the hair follicles. Moreover, strong reactivity of the meibomian glands was observed. Reactivity was present in the meibocytes and in the epithelial cells of the excretory ducts of the meibomian glands. In contrast, psoriasin reactivity was absent in the glands of Moll.

Psoriasin Expression Is Induced by Proinflammatory Mediators and Bacterial Components in Cultivated HCE Cells

To investigate the regulation of psoriasis under conditions such as inflammation and bacterial colonization, we studied its expression in cultivated SV-40-transformed HCE cells treated with inflammatory cytokines, growth factors, and bacterial components and products. Confluent monolayers of cultured HCE cells were treated with IL-1β, TNF-α, VEGF, LPS, PG, and supernatants of S. aureus (Sa), H. influenzae (Hi), and E. coli (Ec) for 24 hours. Psoriasin mRNA was expressed by both untreated (Fig. 5) and treated HCE cells. Real-time RT-PCR results revealed induction of psoriasin transcript after stimulation with 100 ng/mL LPS; 20 ng/mL IL-1β, TNFα, VEGF, and 1:100 dilution of the supernatants of S. aureus (Sa) and H. influenza (Hi) (Fig. 5A). The highest induction was observed on administration of the proinflammatory cytokine IL-1β (11.8 ± 1.9-fold) compared with that in the unstimulated cells (medium). Basal psoriasin mRNA levels increased up to 9.6 ± 2.5- and 3.9 ± 0.6-fold after treatment with VEGF and TNFα. Furthermore, bacterial products, such as LPS (3.2 ± 1.0-fold) and supernatants of S. aureus (8.3 ± 1.7-fold) and H. influenza (8.4 ± 2.1-fold) upregulated psoriasin transcript in cultivated HCE cells. No significant change was observed after treatment.

![Figure 2](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933461/)

**FIGURE 2.** Expression of psoriasin protein in cultivated ocular surface epithelial cells and tissues of the lacrimal apparatus. Western blot analysis of human psoriasin derived from the following cultivated cells and cadaveric tissues (n = 4 for each tissue): human corneal epithelial (HCE) cells, human conjunctival epithelial (HCJE) cells, lacrimal glands (lg), nasolacrimal ducts (nd), conjunctivae (cj), and cornaeae (co). Proteins were separated by SDS-PAGE and show distinct bands for psoriasin (11 kDa). Controls were performed without psoriasin antibody (O) and with tissue from eye lid (pc, positive control). β-Actin (42 kDa) was measured as a loading control. Results are representative of those in three additional experiments.

![Figure 3](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933461/)

**FIGURE 3.** Psoriasin concentration in several cadaveric tissues of the lacrimal apparatus and in the tear fluid of healthy volunteers was examined by psoriasin ELISA. Total protein of conjunctiva (n = 4), lacrimal gland (n = 7), and nasolacrimal duct (n = 4) was extracted, and psoriasin concentrations were determined by ELISA. Tear fluid obtained from healthy volunteers (n = 4) was also examined by psoriasin ELISA. Each bar represents the mean ± SEM of each tissue and tear fluid.
with 100 ng/mL PG or the supernatant of *E. coli* (1:100 dilution).

To gain more insight into gene regulation of psoriasin, we conducted further stimulation experiments. Stimulation of HCE cells for 24 hours with different concentrations of VEGF, a growth factor that stimulates the growth of new blood vessels in vivo, demonstrated a transcriptional upregulation of the psoriasin gene. VEGF stimulation revealed a concentration-dependent increase in psoriasin gene expression after treatment with 5 (4.2 ± 1.2-fold), 10 (6.3 ± 0.4-fold), 20 (9.6 ± 2.5-fold), and 50 (29.7 ± 4.8-fold) ng/mL, compared with expression in untreated cells (Fig. 5B).

To further analyze the observed upregulatory effect of proinflammatory cytokines on psoriasin mRNA, we probed the effect of different IL-1β and TNFα concentrations (5, 20, and 50 ng/mL). Moreover, the duration of treatment (6, 12, and 24 hours) with IL-1β on the mRNA level of psoriasin was tested as well. Real-time RT-PCR indicated an upregulation in the psoriasin gene expression after stimulation with both cytokines in cultivated HCE cells. IL-1β strongly induced psoriasin expression, whereas a weaker effect was seen after stimulation with TNFα (Figs. 5C, 5D). Psoriasin mRNA expression increased after stimulation (24 hours) with 5 (20.8 ± 3.6-fold), 20 (11.8 ± 1.9-fold) and 50 (8.3 ± 1.9-fold) ng/mL IL-1β compared with nonstimulated cells. TNFα stimulation also caused a significant increase in psoriasin expression after treatment with 5 (5.2 ± 0.8-fold) and 20 (3.9 ± 0.6-fold) ng/mL, whereas a higher TNFα concentration (50 ng/mL) had no effect.

Our results further indicated that psoriasin regulation in the HCE cells was a time-dependent process. Treatment with IL-1β for several time points demonstrated a significant upregulation of psoriasin transcription after 6 hours with a maximum after 12 hours. At 24 hours, the psoriasin mRNA level was in decline, but remained continuously high level.

**Supernatant of *S. aureus* Induces Psoriasin Expression in HCE Cells**

As our results demonstrated that products of bacteria and supernatants of bacteria which are frequently involved in ocular surface infection affect the expression of human psoriasin in cultured HCE cells (Fig. 5A), we tested in further real-time RT-PCR experiments (Fig. 6) the inducibility of psoriasin gene expression in cultured HCE and HCJEp1 cells after challenge with total bacterial culture supernatants. To this end, supernatants of the Gram-positive bacterium *S. aureus* and Gram-negative bacterium *H. influenzae* were analyzed at different time points. After stimulation with the supernatant of *H. influenzae* (1:100), the basal psoriasin mRNA level increased 1.6-fold in cultured HCE cells and 2.2-fold in HCJEp1 cells, compared with unstimulated cells (medium alone). In contrast, treatment with supernatant of *S. aureus* (1:100) revealed a threefold upregulation of psoriasin transcript in cultivated HCE cells. No induction was detected in the HCJEp1 cells.

To investigate the influence of different incubation time points, we treated HCE cells for 4, 6, 12, 16, 24, and 48 hours with supernatants of *H. influenzae* (1:100) and *S. aureus* (1:100), and psoriasin expression was again examined with real-time RT-PCR (Fig. 7). Results indicated an upregulation of psoriasin transcript at 4 to 24 hours after stimulation with the supernatant of *S. aureus*. Basal psoriasin mRNA levels increased nearly threefold during a 24-hour stimulation, with a maximum peak after 16 hours (4.4 ± 1.2-fold). No psoriasin induction was detected after 48 hours of treatment in HCE cells. Stimulation with the supernatant of *H. influenzae* increased psoriasin expression by approximately 2- to 2.5-fold after 16 hours, with a maximum peak at 48 hours (3.2 ± 0.2-fold). No induction was detected before 16 hours of *H. influenzae* treatment.

**FIGURE 4.** Immunohistochemical detection of psoriasin in tissues of the human lacrimal apparatus. Images are representative of human cornea (A), conjunctiva (B), lacrimal glands (C), nasolacrimal ducts (D), and meibomian glands of the upper eyelid (E). Immunostaining was performed with antibody prepared against human psoriasin. Positive control sections from hair follicle and squamous epithelium (F). Positive reactivity stained red (arrows and asterisks). Scale bars: (A–C) 10 μm; (D) 40 μm; (E, F) 80 μm.
DISCUSSION

The human ocular surface epithelia and epithelial cells of the lacrimal apparatus express several cationic antimicrobial peptides, including human β-defensins (hBD) and human cathelicidin (LL-37) as part of the innate immune system. In the present study, we characterized the expression of psoriasin as a further antimicrobial peptide, to expand the knowledge about the innate immune responses at the ocular surface.

Psoriasin, originally isolated as an overexpressed molecule of unknown function from psoriasis keratinocytes, has recently been identified as a principal *E. coli*-killing antimicrobial peptide of healthy skin as well as some mucosal surfaces, such as tongue and the female genital tract. At the ocular surface, psoriasin is constitutively expressed in cornea, conjunctiva, lacrimal glands, and nasolacrimal ducts on the mRNA and protein levels. Psoriasin transcripts were detected in all analyzed ocular tissue samples. The simultaneous amplification of the housekeeping gene β-actin allowed a subjective quantitative correlation of psoriasin gene expression between different ocular surface tissues and tissues of the lacrimal apparatus. Psoriasin mRNA was expressed in equivalent amounts in human conjunctiva, lacrimal gland, and nasolacrimal ducts. Only in human cornea was the expression rate lower. Western blot analysis with monoclonal psoriasin antibodies revealed a single band at 11 kDa in all investigated tissues and verified the mRNA results. For control of RT-PCR and Western blot experiments, human skin was used, where the presence of the psoriasin gene and peptide has already been described. These controls revealed clear positive signals in all experiments. Immunohistochemistry localized psoriasin in the squamous epithelium (stratum granulosum and stratum corneum) and around the hair follicles of the eyelid. In addition, strong immunoreactivity was visible in the meibocytes of meibomian glands which are anatomically specialized, free sebaceous glands without hair. In contrast, psoriasin reactivity was not detected in the eccrine sweat glands of Moll, which have been shown to produce β-defensins and cathelicidin. Immunohistochemistry of human lacrimal glands revealed that psoriasin was produced in 11 of 18 samples. In the presence of positive reactivity, psoriasin was visible intracytoplasmically in acinus cells in some but not all acini (Fig. 3), suggesting that the lacrimal gland is not the main source of tear film psoriasin but can contribute to psoriasin production. Whether psoriasin was induced in positively stained lacrimal glands, whether the concentration was below the detection limits in the nonreactive lacrimal glands or whether only parts of a lacrimal gland produce psoriasin must be determined. Furthermore, partial degradation of psoriasin in the lacrimal

FIGURE 5. Regulation of psoriasin mRNA expression in HCE cells after challenge with cytokines and bacterial compounds. Real-time data represent the fold-increase in psoriasin transcript levels versus untreated cells (medium). Each bar represents the mean ± SEM of four independent experiments. (A) HCE cells were stimulated with 100 ng/mL peptidoglycan (PG), lipopolysaccharide (LPS), 20 ng/mL of interleukin-1β (IL-1β), tumor necrosis factor (TNF)-α or vascular endothelial growth factor (VEGF) as well as 1:100 dilution of supernatant of *S. aureus* (Sa), *H. influenza* (Hi) and *E. coli* (Ec) for 24 hours. (B) Stimulation of HCE cells with different VEGF concentration for 24 hours. (C) Cultivated HCE cells were treated for 24 hours with different concentrations of IL-1β and TNFα. (D) Stimulation with IL-1β over different time points. Each real-time experiment was run in triplicate. *P < 0.05, **P < 0.01, ***P < 0.001 by unpaired, two-tailed Student’s *t*-test.
gland may be possible because the variable postmortem time frame of the cadavers could lead to loss of reactivity. RT-PCR and Western blot investigations confirmed these findings in human lacrimal glands. Gläser et al. measured different psoriasin amounts at the skin surface depending on the donor. Moreover, we found cytoplasmic staining of psoriasin in high columnar epithelial cells of the epithelial layer of the conjunctiva and nasolacrimal ducts. The secretory products of goblet cells showed no immunoreactivity. Sections of human cornea demonstrated only a weak reactivity in superficial corneal epithelial cells, whereas no immunohistochemical reaction was observed in endothelial or stromal cells of healthy cornea. Psoriasin may be inducible in these corneal layers, as it has been observed with surfactant proteins A and D, two proteins that contribute to the innate immune response of the ocular surface, that are not visible in the deeper corneal layers under healthy conditions, but that are induced in cases of bacterial or viral corneal infection. All these findings indicate that psoriasin seems to be an important component of the chemical barrier of the ocular surface and lacrimal apparatus.

In addition to the tissues investigated, we detected psoriasin in human tear fluid obtained from healthy donors by ELISA. Of note, the highest amount of psoriasin was detected in the tear fluid (up to 175 ng/mg total protein), whereas cadaveric tissue samples contained clearly lower psoriasin concentrations. Gläser et al. measured psoriasin amounts at the skin surface mainly in the range of 5 to 20 ng/cm², with peak amounts up to 100 ng/cm² in skin areas where microbe densities are physiologically high and where the epidermis is rich in sebaceous glands. Several other antimicrobial peptides have been reported in tear fluid and/or are produced by corneal and conjunctival epithelium (for review, see McDermott et al.). In a previous study, we were able to determine the concentration of hBD-2 and -3, which are well described at the ocular surface, at a much lower level (hBD-2 386.8 pg/mg total protein; hBD-3 141.8 pg/mg total protein). This high psoriasin concentration in tear fluid under normal healthy conditions might explain the very rare infection of the human ocular surface with E. coli.

It has been shown in a wide variety of interesting studies that different S100 calcium binding proteins (S100) are upregulated in cases of pterygium and Sjögren’s syndrome as well as...
in the tear fluid identifying them as biomarkers of dry eye syndrome.\textsuperscript{44–47} Particularly in S100A8 (calgranulin A) and S100A9 (calgranulin B), which have been described as multifunctional inflammatory response proteins, expression has been observed to increase in patients with meibomian gland dysfunction (MGD), a major cause of dry eye syndrome.\textsuperscript{48} It has been further proposed that increased S100A8/A9 gene expression, in combination with upregulation of various small proline-rich proteins (SPRR), is responsible for the hyperkeratinization of meibomian gland ductal epithelium, which is the initial process in MGD.\textsuperscript{49} Of note, the large amount of data obtained by Liu et al.\textsuperscript{50} also revealed psoriasin (S100A7) to be significantly upregulated, with a ratio of 7.33, making it the third most upregulated gene in the human meibomian gland in cases of MGD. This finding is very consistent with our presented psoriasin (S100A7) data, especially the strong immunoreactivity of psoriasin in meibocytes and ductal epithelium of the meibomian glands. It will be of interest to analyze the functional role of psoriasin in other ocular surface diseases.

Besides human tissue samples of the ocular surface and lacrimal apparatus we observed expression of psoriasin in cultured HCE and HCjE cells. This finding allowed the investigation of psoriasin regulation under such conditions as inflammation and bacterial colonization in vitro. Both bacteria and their PAMPs lead to the induction of the innate immune system triggered by the production of proinflammatory cytokines such as IL-1β or TNFα.\textsuperscript{50} We showed that gene expression of psoriasin in HCE cells was upregulated by IL-1β and to a lesser extent by TNFα. Quantitative real-time RT-PCR data indicated an upregulation of psoriasin gene in a time- and concentration-dependent manner. Similar results were described in primary keratinocytes under simulated inflammatory conditions.\textsuperscript{19} Cultivated HCE cells were stimulated with VEGF, which plays a key role in angiogenesis and is also induced under inflammatory conditions and during tumorigenesis (for review, see Sung et al.).\textsuperscript{17} Our results revealed a significant increase in psoriasin gene expression (30-fold; 50 ng/ml VEGF for 24 hours). Work is under way to address the question of whether psoriasin has a regulatory function in (anti)angiogenesis at the ocular surface.

In the present study, we also found that bacterial compounds and inactivated bacterial supernatant of \textit{S. aureus} and \textit{H. influenzae} induced psoriasin expression in cultivated HCE and HCjE cells. LPS, a major component of the outer membrane of Gram-negative bacteria which activates Toll-like receptor (TLR)-4, induced psoriasin expression in HCE cells. Gram-positive \textit{S. aureus} and Gram-negative \textit{H. influenzae} are common ocular pathogens that are involved in bacterial keratitis.\textsuperscript{13} In cultivated HCE cells, psoriasin mRNA expression was upregulated after treatment with supernatants of \textit{S. aureus} and \textit{H. influenzae}. In HCjE cells, psoriasin mRNA induction was detected only after stimulation with the supernatant of \textit{H. influenzae} but not with that of \textit{S. aureus}. Our results indicate that psoriasin mRNA expression was upregulated at different time points after stimulation with \textit{S. aureus} and \textit{H. influenzae}. The psoriasin gene was upregulated from 4 to 24 hours after treatment with the \textit{S. aureus} supernatant and from 16 to 48 hours after stimulation with \textit{H. influenzae} supernatant in cultivated HCE cells. Gläser et al.\textsuperscript{19} showed potent antimicrobial activity of psoriasin against several \textit{E. coli} strains and far less activity against \textit{S. aureus}. The antimicrobial activity against \textit{H. influenzae} was not tested before. In both cell lines used in the present study no psoriasin induction was detected after stimulation with supernatants from \textit{E. coli}. This is surprising, because in primary and cultured keratinocytes, a strong psoriasin induction after treatment with different supernatants of various \textit{E. coli} strains has been described and psoriasin also belongs to the innate immune system acting against \textit{E. coli} colonization and infection at human skin surface.\textsuperscript{18,54} A possible explanation is that we used an \textit{E. coli} strain (ATCC 8739) that has no pathogenic potential to stimulate the psoriasin expression cascade or that one or more other antimicrobial peptides took on the role of psoriasin in the innate immune response at the ocular surface that are so far not known. In this context, it is known that human β-defensins and human cathelicidin LL-37 have a good activity against different Gram-positive and -bacteria, such as \textit{S. aureus} or \textit{E. coli} (for review see McDermott\textsuperscript{75}). Also, synergistic interaction between AMPs and other antimicrobial molecules like lysozyme have been observed in different epithelial layers.\textsuperscript{55–57}

In conclusion, we have shown that the antimicrobial peptide psoriasin is part of the innate immune responses at the ocular surface and the lacrimal apparatus. Psoriasin is induced by proinflammatory cytokines as well as bacterial compounds at least in HCE and HCjE cells and seems to have a regulatory function with regard to (anti)angiogenesis at the ocular surface. Our results notably confirm that psoriasin is part of an additional reservoir of lipophilic antimicrobial peptides at the ocular surface as especially meibocytes are a side of psoriasin production. The breakdown of meibomian gland function\textsuperscript{58} could be a reason for the increase in ocular surface inflammation in some cases of dry eye, as, with meibomian gland dysfunction, psoriasin production and its antimicrobial protection are also reduced.

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References


