Detection of Surfactant Proteins A and D in Human Tear Fluid and the Human Lacrimal System

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Purpose. To evaluate the expression and presence of surfactant protein (SP) A and SP-D in the lacrimal apparatus, at the ocular surface, and in tears in healthy and pathologic states.

Methods. Expression of mRNA for SP-A and SP-D was analyzed by RT-PCR in healthy lacrimal gland, conjunctiva, cornea, and nasolacrimal ducts as well as in a spontaneously immortalized conjunctival epithelial cell line (HCjE; IOBA-NHC) and a SV40-transfected cornea epithelial cell line (HCE). Deposition of SP-A and SP-D was determined by Western blot, dot blot, and immunohistochemistry in healthy tissues, in tears, aqueous humor, and in sections of different corneal abnormalities (keratoconus, herpetic keratitis, and *Staphylococcus aureus*–based ulceration). Cell lines were stimulated with different cytokines and bacterial components and were analyzed for the production of SP-A and SP-D by immunohistochemistry.

Results. The presence of SP-A and SP-D on mRNA and protein levels was evidenced in healthy lacrimal gland, conjunctiva, cornea, and nasolacrimal duct samples. Moreover, both proteins were present in tears but were absent in aqueous humor. Immunohistochemistry revealed the production of both peptides by acinar epithelial cells of the lacrimal gland and epithelial cells of the conjunctiva and nasolacrimal ducts, whereas goblet cells revealed no reactivity. Healthy cornea revealed weak reactivity on epithelial surface cells only. In contrast, SP-A and SP-D revealed strong reactivity in patients with herpetic keratitis and corneal ulceration surrounding lesions and in several immigrated defense cells. Reactivity in corneal epithelium and endothelium was also seen in patients with keratoconus. Cell culture experiments revealed that SP-A and SP-D are produced by both epithelial cell lines without and after stimulation with cytokines and bacterial components.

Conclusions. These results show that SP-A, in addition to SP-D, is a peptide of the tear film. Based on the known direct and indirect antimicrobial effects of collectins, the surfactant-associated proteins A and D seem to be involved in several ocular surface diseases. (*Invest Ophthalmol Vis Sci.* 2007;48:3945–3953) DOI:10.1167/iovs.07-0201

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The eye has a variety of natural barrier defenses against external stimuli. Lids, eyelashes, and eyebrows protect against light, wind, and particulate matter. What succeeds in touching the ocular surface is buffered by the tear film, the most crucial barrier the eye has against particulate matter, such as pollutants, pollen, dust, mold, mite fecal particles, animal dander, and other proteins, as well as dirt and sand. A normally functioning tear film is of outstanding importance to the eye's defenses against these intruders and also ensures an optimal refractive surface. Today it is generally accepted that the preocular fluid is a complex secretion with structure on all scales: lipids, an aqueous component dissolving a great variety of chemical entities, and mucins contribute to a stable and continuous layer covering the external ocular epithelia and anchored onto their apical surfaces. The lipid component originates from the Meibomian glands of the tarsus (McCulley and Shine,[1] Sullivan et al.[2]). The mucus component of the tear film is formed by giant glycoconjugates called mucins that originate as gel-forming mucins from conjunctival goblet cells (for a review, see Gipson et al.[3]) and acinar cells of the human lacrimal gland[4,5] and as membrane-associated mucins from human corneal and conjunctival epithelial cells.[6,7] The aqueous component contains electrolytes, water, and a large variety of proteins, oligopeptides, and glycopeptides and is primarily secreted by the main lacrimal and accessory lacrimal glands. The acini of the lacrimal gland comprise a laminar lining of columnar epithelial cells surrounded by a basal layer of myoepithelial cells and an enclosing basement membrane. Intercalated and interlobular ducts drain the secretions into the conjunctival space beneath the temporal upper eyelid.

Surfactant protein (SP) A and SP-D are members of the collectin family of C-type lectins that includes a number of molecules with known host defense functions. According to the current notion, collectins bind to carbohydrates expressed on the surfaces of various microorganisms and to specific receptors on phagocytotic cells, thus accelerating microbial clearance.[8,9] Individual collectins and their specific structural domains seem to play specific roles in host defense.[10]

SP-A is a 28- to 36-kDa protein expressed primarily by type 2 alveolar epithelial cells in the lung. SP-A isolated from bronchoalveolar lavage fluid is mainly bound to surfactant lipid aggregates. SP-A improves the SP-B–mediated surface tension reducing properties of surfactant lipids. However, deletion of the SP-A encoding gene apparently does not affect lung stability.[11] SP-A binds to pulmonary type 2 alveolar cells and immune cells, particularly alveolar macrophages.[12] The proposed functions of SP-A in vitro include the enhancement of surface activity,[13,14] maintenance of homoeostasis between the extracellular and intracellular surfactant pools,[15] and involvement in non-antibody–mediated defense against microorganisms.[12] Furthermore, SP-A knockout mice have been shown to have reduced defenses against several lung pathogens.[16,17]

SP-D is a 43-kDa protein synthesized and secreted by pulmonary alveolar type 2 cells, nonciliated airway cells, and cells of the gastric mucosa. It shares considerable homology with SP-A and other mammalian lectins.[18–20] SP-D binds carbohydrates and lipids in a calcium-dependent manner and probably plays a role in innate host defense against various bacterial, fungal, and viral pathogens. SP-D interacts with a number of microorganisms, including influenza A virus[21] and bacteria, among them *Pseudomonas aeruginosa* and *Escherichia coli*.[19,22–25] SP-D binds to the core antigens in LPS in a manner distinct from SP-A binding. Targeted deletion of murine SP-D[26,27] has demonstrated its important role in lipid homoeostasis in the lung. SP-D null mice developed alveolar lipidosis associated with activation of alveolar macrophages, increasing oxidant production, and metalloproteinase activation.[28] SP-D–deficient mice also developed an emphysema-like abnormality, demonstrating a critical role of surfactant proteins in the regulation of pulmonary inflammation and remodeling.[28] In addition, SP-D–deficient mice were highly susceptible to infection by influenza A virus in vivo.[29]

Although most studies regarding the function of SP-D have focused on its potential role in the lung, SP-D mRNA was detected in various organs, including the lacrimal gland, in human and mice.[30–32] In contrast to SP-D, recent studies[31,33] indicated that SP-A is not produced by mouse and human lacrimal gland or tear fluid. However, as early as 1994, Dobbie et al.[34] demonstrated by immunohistochemistry that, in addition to occurring in a number of different human tissues and fluids, SP-A is localized in the lacrimal gland. Based on these contradictory statements and findings, the aim of our study was to analyze the entire human lacrimal system for its ability to express and produce SP-A and SP-D in healthy states and in the cornea in pathologic states.

METHODS

Tissues and Cell Lines

The study was conducted in compliance with Institutional Review Board regulations, informed consent regulations, and the provisions of the Declaration of Helsinki. Lacrimal glands, upper eyelids, conjunctivas, corneas, and nasolacrimal systems (consisting of lacrimal sac and nasolacrimal duct) were obtained from cadavers (5 male, 8 female, aged 49–88 years) donated to the Department of Anatomy and Cell Biology, Martin-Luther-University Halle-Wittenberg, Germany. All used tissues were dissected from the cadavers within 4 to 12 hours postmortem. Donors were free of recent trauma, eye and nasal infections, and diseases involving or affecting lacrimal function.

Twenty-one samples of the central cornea of patients with keratoconus (10 samples), herpetic keratitis (6 samples), and *Staphylococcus aureus*–based ulceration (5 samples) were obtained during surgical procedures at the Department of Ophthalmology, Christian Albrecht University of Kiel, Germany. Medical histories of the patients concerning other possible eye-affecting diseases could be obtained. Two patients with keratoconus had neurodermatitis.

After dissection, tissues from the right eye of each cadaver were prepared for paraffin embedding and were fixed in 4% paraformaldehyde; tissues from each left eye were used for molecular biological investigations and were immediately frozen at –80°C. After surgery, all corneae from patients were fixed in 4% formalin, dehydrated in graded concentrations of ethanol, and embedded in paraffin.

SV40-transformed human corneal epithelial (HCE) cells, a kind gift from Kaoru Araki-Sasaki (Tane Memorial Eye Hospital, Osaka, Japan),[35] and a human, spontaneously immortalized epithelial cell line from normal human conjunctiva (IOBA-NHC, here referred to as HCjE cells; a kind gift from Yolanda Diebold, University Institute of Applied Ophthalmobiology [IOBA], University of Valladolid, Valladolid, Spain)[36] were cultured as monolayers and used for stimulation experiments.

Collection of Tear Fluid and Aqueous Humor

Tear fluid was collected from the lower conjunctival sacs of six healthy human volunteers by the capillary tube method, as previously described by others.[37] A tear volume of 100 μL was collected over approximately 10 minutes on each occasion. The large amount of obtained tear fluid within the short time-frame is indicative of reflex tears. Collected tear samples of each volunteer were pooled, immediately frozen, and stored at –20°C for use in experiments.

We included aqueous humor of six patients who underwent regular phacoemulsification for cataract removal (Charité, Department of Ophthalmology, Berlin). Only eyes without any other abnormality, particularly inflammatory history, were included. Anterior chamber puncture was performed under local anesthesia by introducing a 30-gauge needle through the anterior chamber at the limbus. Approximately 100 to 300 μL aqueous humor was withdrawn, transferred to the laboratory, and preserved at –80°C.

Cell Culture

HCE and HCjE cells (5×10^6) were seeded into 25-cm^2 flasks and were cultured in Dulbecco modified Eagle medium (DMEM F12; PAA Laboratories GmbH, Pasching, Austria) containing 10% FCS (Biochrom AG, Berlin, Germany). Near confluence, the cells were cultured in serum-free DMEM with 0.05% bovine serum albumin for 24 hours. Cells were used for RT-PCR.

HCE cells and HCjE cells were also seeded on glass cover slides in six-well culture plates and were incubated. Near confluence, cells were exposed to IL-1α (10 ng/mL), IL-1β (10 ng/mL), TNFα (10 ng/mL), INF-γ (10 ng/mL), LPS (10 ng/mL), or PGN (1 μg/mL) in serum-free DMEM with 0.05% bovine serum albumin for 24 hours (all values are given as final concentration). All experimental procedures were performed under normoxic conditions (20% PO_2, 5% CO_2). At the end of each experiment, the cells on glass cover slides were fixed for 5 minutes in 4% paraformaldehyde and were processed for immunohistochemistry.

RNA Preparation and Complementary DNA Synthesis

For conventional reverse transcription–polymerase chain reaction (RT-PCR), tissue biopsy samples from 14 lacrimal glands, upper eyelids, conjunctivas, corneas, and nasolacrimal systems were crushed in an agate mortar under liquid nitrogen, then homogenized in 5 mL RNA pure solution (peqGOLD Total RNA Kit; peqLab Biotechnologie, Erlangen, Germany) with a homogenizer (Polytron, Norcross, GA). Insoluble material was removed by centrifugation (12,000*g*, 5 minutes, 4°C). Total RNA was isolated by RNA purification (RNeasyKit; Qiagen, Hilden, Germany). Crude RNA was purified with isopropanol and repeated ethanol precipitation, and contaminating DNA was destroyed by digestion with RNase-free DNAse I (20 minutes 25°C; Boehringer, Mannheim, Germany). Contamination of the purified RNA by genomic DNA was prevented by performing PCR with the specific primers for SP-A and SP-D and for β-actin. In no case was amplification obtainable using the purified RNA as a template. The DNAse was heat denatured for 15 minutes at 65°C. Five hundred nanograms RNA was used for each reaction: cDNA was generated with 50 ng/μL (20 pmol) oligo $(dT)_{15}$ primer (Amersham Pharmacia Biotech, Uppsala, Sweden) and 0.8 μL superscript RNase H^- reverse transcriptase (100 U; Gibco, Paisley, UK) for 60 minutes at 37°C. 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.

PCR

For conventional PCR, 1 μL cDNA (from each sample) was incubated with 13.7 μL H_2O, 1 μL 50 mM $MgCl_2$, 0.5 μL dNTP, 2 μL 10× PCR buffer, 0.3 μL (5 U) *Taq* DNA polymerase (Invitrogen), and 0.5 μL (100 pmol) of each of the following primers: SP-A sense, 5′-GAT GGG CAG

TGG AAT GAC AGG-3′; SP-A antisense, 5′-GGG AAT GAA GTG GCT AAG GGT G-3′ (212 bp); SP-D sense, 5′-AGG AGC AAA GGG AGA AAG TGG G-3′; SP-D antisense 5′-CAG CTG TGC CTC CGT AAA TGG-3′ (199 bp). After 5 minutes of heat denaturation at 96°C, the PCR cycle was conducted at 96°C for 60 seconds, 57°C (SP-A and SP-D) for 60 seconds each, and 72°C for 60 seconds. Thirty-five cycles were performed with each primer pair. The final elongation cycle consisted of 72°C for 4 minutes. Primers were synthesized by MWG-Biotech AG (Ebersberg, Germany). Ten microliters PCR was loaded on an agarose gel. After electrophoresis, the amplified products were visualized by fluorescence. Base pair (bp) values were compared with GenBank data.[38] For verification and comparison, bacterial plasmids carrying the genes for the investigated proteins were used as a reference (German Resource Centre for Genome Research GmbH, Berlin, Germany; SP-A, IRAUp969H0686D6; SP-D, IRAUp969D0386D6). PCR products were also confirmed by sequencing (BigDye; Applied Biosystems, Foster City, CA). To estimate the amount of amplified PCR product, we performed a GAPDH PCR with specific primers for each investigated tissue. For this additional PCR, we used the conditions described. Ten microliters PCR product was loaded on the agarose gel.

Antibodies

Antibodies used were as follows: rabbit anti–human SP-A antibody (Laboratory of Samuel Hawgood, Cardiovascular Research Institute and Department of Pediatrics, University of California San Francisco, San Francisco, CA)[39]; mouse anti–human surfactant protein A (SP-A) monoclonal antibody (MAB3270; Chemicon, Hampshire, UK); rabbit anti–human SP-D antibody (generously provided by Erika Crouch, Washington University, St. Louis, MO)[40]; rabbit anti–human SP-D antibody (AB3434; Chemicon); goat anti–human SP-D antibody (C-18 [sc-7708]; Santa Cruz Biotechnology, Heidelberg, Germany); and mouse anti–human SP-D antibody (BM4083; Acris, Hiddenhausen, Germany).

Western Blot Analysis

For Western blot analysis, human tissue from lacrimal gland, conjunctiva, cornea, and nasolacrimal ducts of cadavers (standardized ratio: 100 mg wet weight/400 μm buffer containing 1% SDS and 4% 2-mercaptoethanol) was extracted as described in detail by Kutta et al.,[41] and the protein content was measured with a protein assay based on the Bradford dye-binding procedure (BioRad, Hercules, CA). Total protein (20 μg) was then analyzed by Western blot. Proteins were resolved by reducing 10% SDS-polyacrylamide gel electrophoresis, electrophoretically transferred at room temperature for 1 hour at 0.8 mA/cm^2 onto 0.1-μm pore nitrocellulose membranes, and fixed with 0.2% glutaraldehyde in phosphate-buffered saline for 30 minutes. Bands were detected with primary antibodies to SP-A (rabbit polyclonal, 1:4000) and SP-D (rabbit polyclonal; Crouch Laboratory and Chemicon; 1:1000) and for SP-A in tear fluid SP-A mouse anti-human monoclonal, (Chemicon; 1:300) and secondary antibodies (anti–rabbit IgG conjugated to horseradish peroxidase (1:10,000 [DAKO, Glostrup, Denmark]) applying chemiluminescence (ECL-Plus; Amersham-Pharmacia, Uppsala, Sweden).

Immunohistochemistry

For analysis by immunohistochemistry, lacrimal glands, upper eye lids with conjunctiva, corneae, nasolacrimal systems, and corneal lesions were sectioned and dewaxed. Immunohistochemical staining was performed with antibodies to SP-A (Dr. S. Hawgood Laboratory; 1:25) and SP-D (1:25; Santa Cruz Biotechnology). Sections were microwaved for 10 minutes, and nonspecific binding was inhibited by incubation with porcine normal serum (DAKO) 1:5 in Tris-buffered saline [TBS]. Primary antibody was applied overnight at room temperature. Secondary antibodies porcine anti–rabbit for SP-A (1:300; DAKO) and rabbit anti-goat (1:400; DAKO) were incubated at room temperature for at least 4 hours. Visualization was achieved with peroxidase-labeled streptavidin-biotin and diaminobenzidine (DAB) for at least 5 minutes. After counterstaining with hemalum, the sections were mounted in Aquatex (Boehringer, Mannheim, Germany). Two negative control sections were used in each case; one was incubated with the secondary antibody only, the other with the primary antibody only. Sections of human lung were used for positive control. The slides were examined under a microscope (Axiophot; Carl Zeiss, Oberkochen, Germany).

Immunodot Blot

Collected tear fluid samples and aqueous humor samples were centrifuged to remove any cell debris. For immunodot blot analysis, 50 μL tear fluid or aqueous humor was directly applied to a membrane (Hybond-ECL; Amersham Biosciences, Piscataway, NJ) by vacuum using a dot-blot apparatus.[5] Nonspecific binding sites were blocked for SP-A by incubating the membrane in TBS containing 5% nonfat powdered milk for 30 minutes at room temperature and for SP-D by incubating the membrane in BSA for 30 minutes at room temperature. The respective membrane was then incubated overnight at room temperature with anti–human SP-A (1:1000 in powdered milk) or anti–human SP-D (1:50 in BSA; Acris) in TBS containing 5% BSA. After the membrane was washed, it was incubated for 1 hour at room temperature with, respectively, secondary antibody goat anti–rabbit IgG (for SP-A) and goat anti–mouse IgG (for SP-D; Acris), both conjugated to horseradish peroxidase diluted 1:8000 with 5% nonfat powdered milk. Immunoreactivity was visualized with a chemiluminescence detection reagent (Santa Cruz Biotechnology), and the results were documented by digital imaging of the stained membrane.

Results

Expression of SP-A and SP-D in Tissue and Cell Lines

SP-A– and SP-D–specific cDNA amplification products (212 bp and 199 bp) were detected in all lacrimal glands, corneae, conjunctivae, and nasolacrimal ducts (n = 14 for each tissue; Fig. 1). The GAPDH control PCR was positive and of similar amount for all investigated tissues, as expected, and it allowed a quantitative correlation of the amplified product (right bands of the agarose gels). Base pair values were equivalent to the expected DNA products compared with GenBank data.

Western Blot Analysis

Five samples each of human lacrimal gland, cornea, conjunctiva, and nasolacrimal ducts were dissected from cadavers (n = 5). Extracts from these samples were tested for surfactant proteins by Western blot analysis (Fig. 2; unglycosylated forms

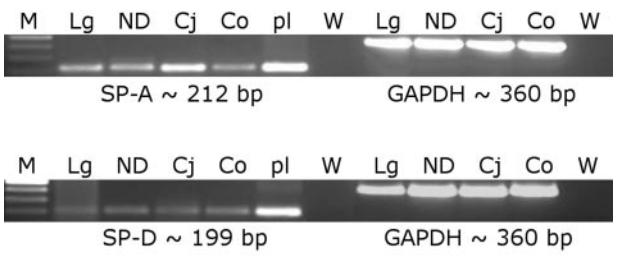

Figure 1. Ethidium bromide–stained agarose gels for visualization of PCR amplification products derived from the following tissues (n = 14 for each tissue): cornea (Co), conjunctiva (Cj), lacrimal gland (Lg), nasolacrimal ducts (ND). PCR amplification products of the bacterial plasmids (pl) were used for positive control; blank lanes (W) indicate the negative control without template DNA. To estimate quantitative correlation, a GAPDH PCR control was performed for each investigated tissue (*right lanes*). In accordance with the DNA marker (M) and the plasmid products, the distinct DNA bands are visible at 212 bp for SP-A and 199 bp for SP-D.

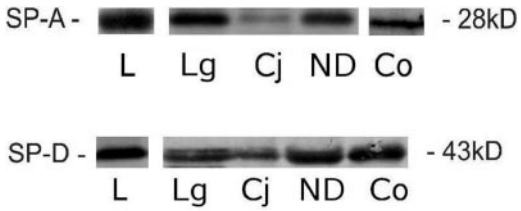

FIGURE 2. Western immunoblots of surfactant proteins SP-A and SP-D derived from the following tissues ($n = 5$ for each tissue): lung (L), lacrimal gland (Lg), conjunctiva (Cj), nasolacrimal ducts (ND), cornea (Co). Proteins were separated by SDS-PAGE under reducing conditions and show distinct bands for SP-A (approximately 28 kDa; unglycosylated form is shown) and SP-D (approximately 43 kDa).

are shown). Varying amounts of SP-A and SP-D were detected in all samples.

Dot Blot Analysis

Tear fluid samples and aqueous humor samples were tested for SP-A and SP-D using dot blot analysis. Both proteins were present in all tear samples but were not detectable in all samples of aqueous humor (Fig. 3A). To improve and verify the results of the dot blot, we performed an additional Western blot with tear fluid of reflex tears for SP-A, revealing the presence of this protein in a distinct band at the same molecular weight in comparison with the control (lung) (Fig. 3B). For this Western blot, we used a monoclonal mouse anti–human antibody from Chemicon (1:300). Detection of SP-D in tear fluid by means of Western blot analysis has already been demonstrated by Ni et al.[33]

Distribution of SP-A and SP-D in Cadaveric Tissue and Cell Lines

Paraffin-embedded 7-μm sections from 13 lacrimal glands, corneae, conjunctivae, and nasolacrimal ducts were analyzed. Control sections (secondary antibody only) were negative (unstained) for each of the investigated tissues (healthy or pathologic).

Lacrimal Gland. Clear positive reactivity was visible intracytoplasmically in acinar cells of all sections analyzed for SP-A and SP-D (Figs. 4A, 4D). Reactivity was especially positive near the lumen of the acinar cells (Figs. 4A, 4D, insets).

Cornea. All corneal sections revealed positive staining for SP-A and SP-D at the surfaces of the most superficial layers of corneal epithelial cells (Figs. 4B, 4E), whereas deeper epithelial cell layers, corneal stroma, and corneal endothelium revealed no reactivity for the two surfactant proteins (Figs. 4C, 4F). Some corneal samples showed hyperplastic alteration of the epithelial cells (Fig. 4E), which might have resulted from undiagnosed disease or surgery of the donors.

Conjunctiva. All sections of conjunctiva revealed strong positive intracytoplasmic reactivity for SP-A and SP-D in the superficial and middle layers of conjunctival epithelial cells (Figs. 4G, 4J). The basal layer did not stain or stained only weakly. Goblet cells showed no reactivity.

Nasolacrimal Ducts. SP-A and SP-D were detected in all efferent tear duct systems investigated. SP-A and SP-D reactivity was visible in the apical part of the high columnar epithelial cells of the double-layered epithelium of the lacrimal sac and nasolacrimal duct (Figs. 4H, 4K). Reactivity was absent in goblet cells and intraepithelial mucous glands but was present in the serous acini and secretion products of subepithelial serous glands of the nasolacrimal passage (Fig. 4K). No differences in SP-A and SP-D distribution were observed between the lacrimal sac and the nasolacrimal duct.

Cell Lines. Positive immunoreactivity was visible in all 8 independent cultured epithelial cell cultures of the two cell lines analyzed before stimulation (Fig. 5, untreated). After stimulation with different cytokines and bacterial components, cultured epithelial cells revealed clear positive staining for SP-A and SP-D (Fig. 5, PGN). Cells showed expression of both surfactant proteins, regardless of whether they were stimulated. Subjectively, the immunohistochemical reaction appeared stronger in stimulated cells than in untreated cells.

Distribution of SP-A and SP-D in Corneal Abnormalities

Keratoconus. In patients with keratoconus, all layers of the epithelium, endothelial cells, and several stromal cells stained positive for SP-A, with the highest concentrations of dye in the epithelial layer, especially supranuclearly and in endothelial cells (Figs. 6A, 6C). The corneal stroma revealed weak reactivity (Fig. 6C). SP-D reactivity was visible in the most superficial layer of epithelial cells and in the basal layer, whereas the middle layers revealed weak reactivity (Figs. 6B,

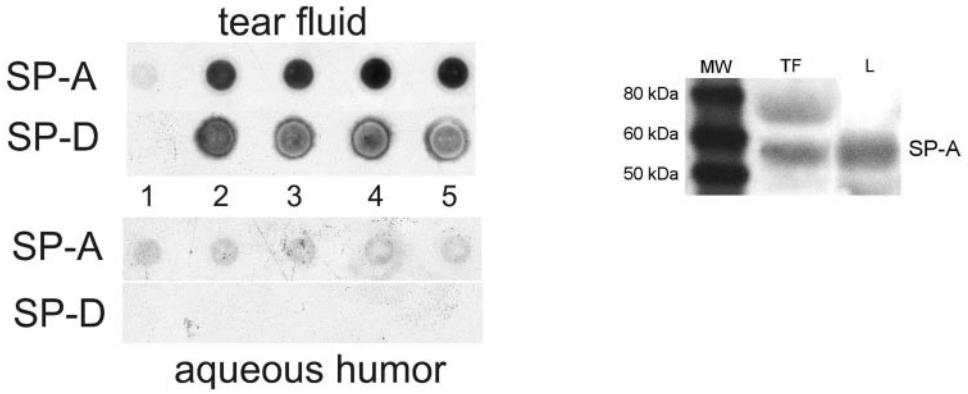

FIGURE 3. (**A**) Dot blot analysis of SP-A and SP-D in tear fluid samples and aqueous humor of four different donors (donors 2–5); 0.1 mg/mL BSA in TBST-buffer was used as negative control (*lane 1*). Aqueous humor samples revealed no reactivity for either surfactant protein. The extra rings in the tear fluid samples for SP-D might have occurred because of leakage. (**B**) Relevant Western blot analysis of tear fluid (TF) using a monoclonal antibody against human SP-A showed a band at the same molecular weight range compared with the positive control (L, lung tissue) at approximately 60 kDa. The *upper band* in the tear fluid lane might have resulted from aggregation of the protein after loading on the gel.

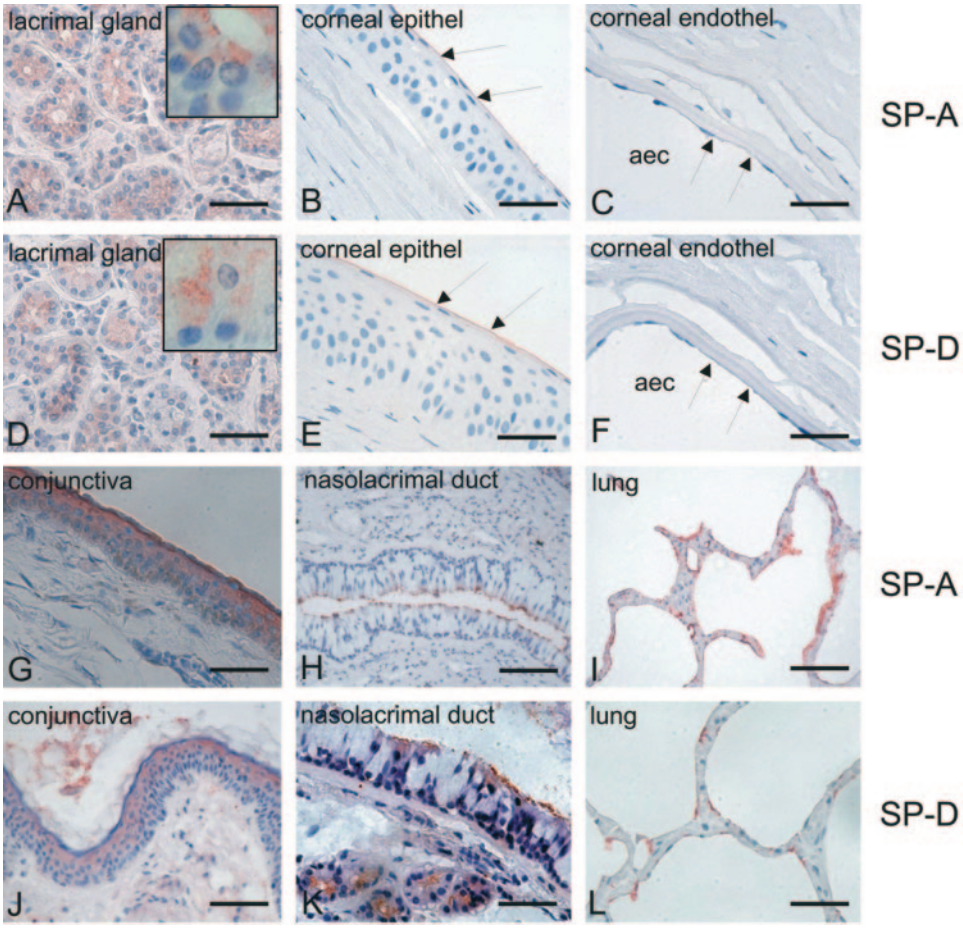

FIGURE 4. (**A**, **D**) Immunohistochemical detection of surfactant proteins. Acinar cells of the lacrimal gland revealed positive reactivity, mainly in the upper part of the cytoplasm, for both SP-A and SP-D. *Insets*: magnified individual acinar cells. Healthy cornea reveals positive staining only in the uppermost layers of epithelial cells (**B**, **E**), whereas the corneal stroma (**B**, **C**, **E**, **F**) and endothelium (**C**, **F**, *arrows*) are negative. The multilayer conjunctival epithelium stained positive for SP-A (**G**) and SP-D (**J**). Positive staining of SP-A (**H**) and SP-D (**K**) in the upper part of high columnar epithelial cells in the nasolacrimal ducts. Subepithelial serous parts of seromucous glands were SP-D reactive (**K**). (**I**, **L**) Positive control sections from human lung, revealing positive red staining in pneumocytes lining alveoli. aec, anterior eye chamber. (**A**–**L**) Hemalum. Scale bars: 56 μm (**A**–**G**, **K**); 82.5 μm (**H**–**J**, **L**).

6D). Some stromal cells and several endothelial cells revealed positive reactivity (Figs. 6B, 6D).

Herpetic Keratitis. Positive staining for SP-A and SP-D was visible in all cases investigated and was especially visible surrounding herpetic lesions (Figs. 7A, 7B). Moreover, several defense cells (Figs. 7A inset, 7D [magnification of 7B in which some defense cells are visible]) that migrated to the corneal epithelium and stroma stained positive for SP-A and SP-D (Figs. 7A, 7B). Epithelial cells themselves (Figs. 7C–F), stromal cells (Figs. 7C, 7D), and endothelial cells (Figs. 7G, 7H) revealed positive staining for both surfactant proteins.

***S. aureus*–Based Ulceration.** Positive reactivity was visible for SP-A and SP-D surrounding the area of the corneal ulcus (Figs. 8A, 8B). Stromal cells were all positive, and the corneal endothelium stained positive (Figs. 8C, 8D). Moreover, many different defense cells stained positive and were located in the corneal stroma (Figs. 8A, 8B).

DISCUSSION

All data obtained in this study demonstrate that, in addition to SP-D, SP-A is present within human tear fluid, tissues of the

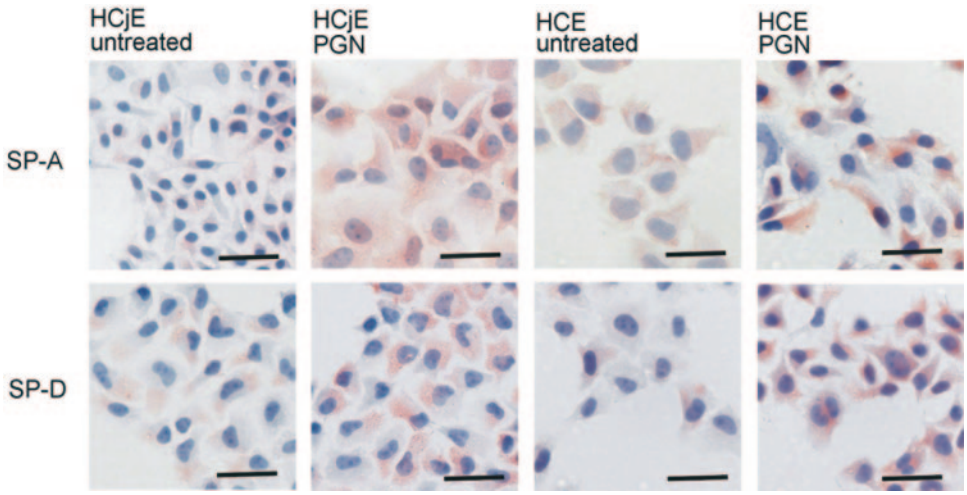

FIGURE 5. Immunohistochemistry of cultured immortalized HCjE and HCE cells. *Red* staining indicates SP-A and SP-D in the cytoplasm of epithelial cells. Images are representative of a series of stimulated and untreated incubated cells. Scale bars: 32.5 μm (**A**); 15 μm (**B**–**H**).

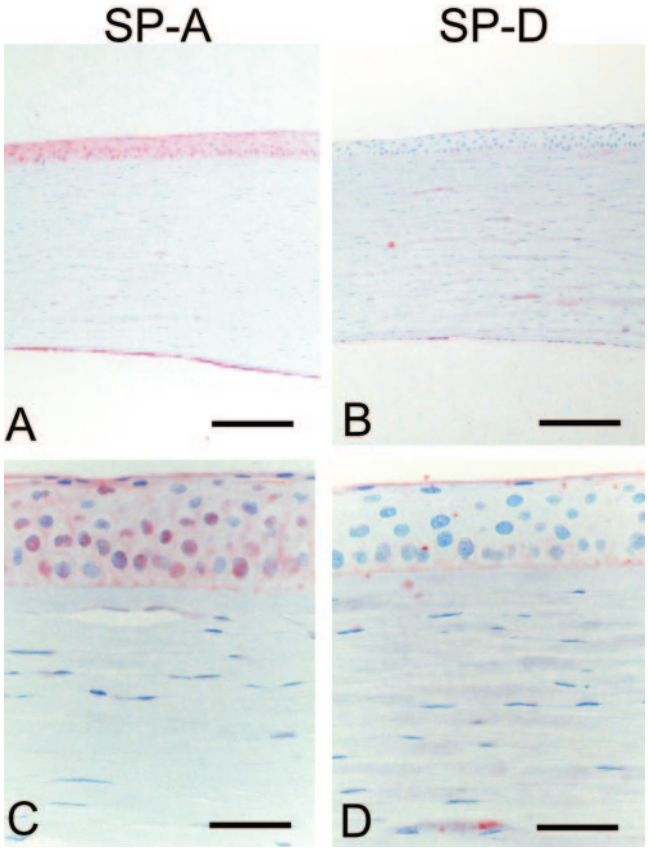

FIGURE 6. Immunohistochemical detection of SP-A and SP-D in a patient with keratoconus. SP-A positivity is visible in the epithelium (**A**, **C**), especially supranuclearly, in several stromal cells, and in the corneal endothelium (**A**). SP-D reactivity is visible on the surfaces of epithelial cells, in the cytoplasm of basal cells (**B**, **D**), in the stroma, and in several endothelial cells. (**A–D**) Hemalum. Scale bars: 32 μm (**A**, **B**); 176 μm (**C**, **D**).

human lacrimal apparatus, and even cultured immortalized human corneal epithelial (HCE) and human conjunctival epithelial (HCjE) cells. Thus, previous findings of Stahlman et al.[31] and Ni et al.[33] about the absence of SP-A in human tear fluid and human lacrimal glands must be revised, and the first description of SP-A in lacrimal gland must be attributed to Dobbie et al.[34] Our results show that SP-A and SP-D are present in the entire human lacrimal apparatus (lacrimal gland, nasolacrimal ducts), conjunctiva, cornea, and human tear fluid. SP-A and SP-D were detected on the mRNA level. The simultaneous amplification of GAPDH allows subjective quantitative correlation of both surfactant proteins and shows, in fact, a detectable level of SP-A and SP-D mRNA for the investigated tissues. Through the application of Western blot and dot blot analysis, the proteins were visualized with specific antibodies. Aqueous fluid seems to contain neither SP-A nor SP-D, whereas human tear fluid reacts with the specific SP-A and SP-D antibodies and reveals the presence of both proteins in tear fluid. Hence, we assumed that surfactant proteins were absent in aqueous fluid or that the concentration was below detection limits. The detected protein bands for tear fluid using a monoclonal antibody for SP-A are characterized by an unexpected higher molecular weight of approximately 60 kDa. Nevertheless, the positive control (lung) in this special case was also detected at the same molecular weight using the same antibody. We assumed that the protein might have been reaggregated to some extent after loading on the gel.

Immunohistochemical localization studies revealed cytoplasmic staining of SP-A and SP-D in acinus cells of the lacrimal gland and in the cytoplasm of high columnar epithelial cells of the lacrimal sac and nasolacrimal duct. Immunohistochemistry also demonstrated reactivity of both proteins on the most superficial layers of corneal epithelial cells, the superficial layers of conjunctival epithelial cells, and in the cytoplasm of cultured HCE and HCjE cells, indicating that both proteins are produced by all these structures. With regard to SP-D, these results were mostly expected because production of SP-D has

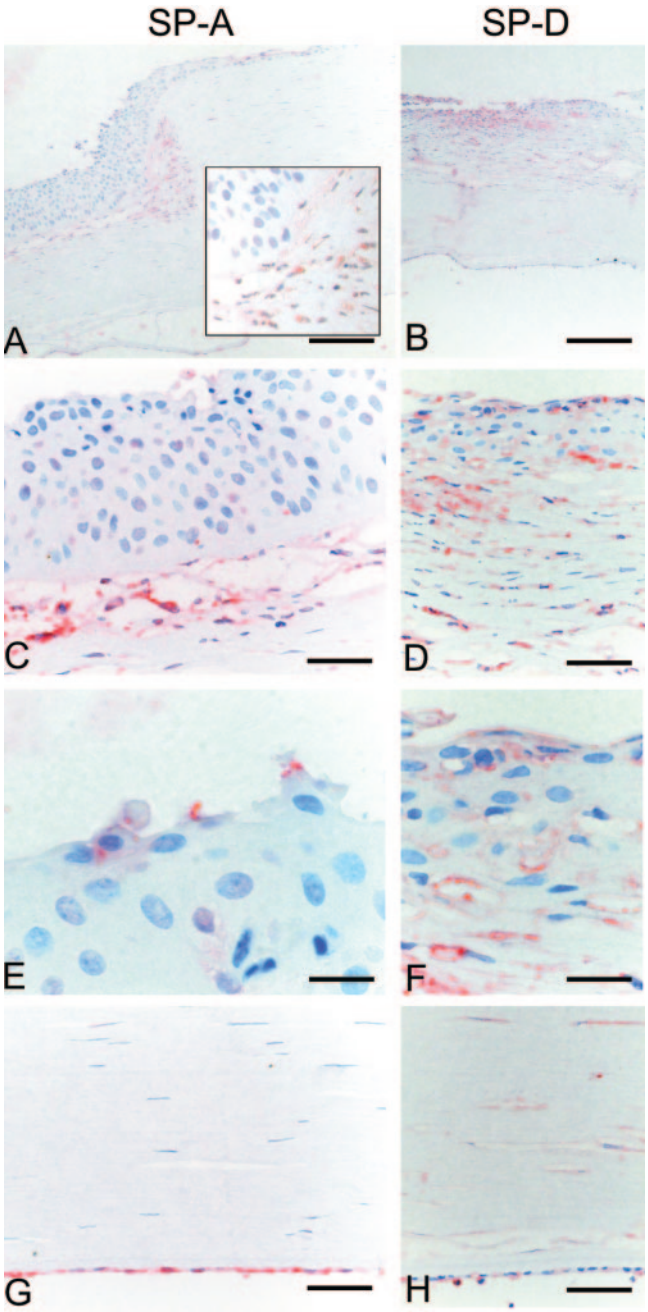

FIGURE 7. Immunohistochemical detection of SP-A and SP-D in herpetic keratitis. Positive reactivity is especially visible surrounding the herpetic lesions (**A**, **B**). (**A**, *inset*) Magnification of defense cells near the lesions. Higher magnification reveals reactivity of both antibodies under the epithelium in stromal cells (**C**, **D**), in epithelial cells (**C**, **D**, **E**, **F**) and in the corneal endothelium (**G**, **H**). (**A–H**) Hemalum. Scale bars: 82.5 μm (**A**, **B**); 56 μm (**C**, **D**, **G**, **H**); 14 μm (**E**, **F**).

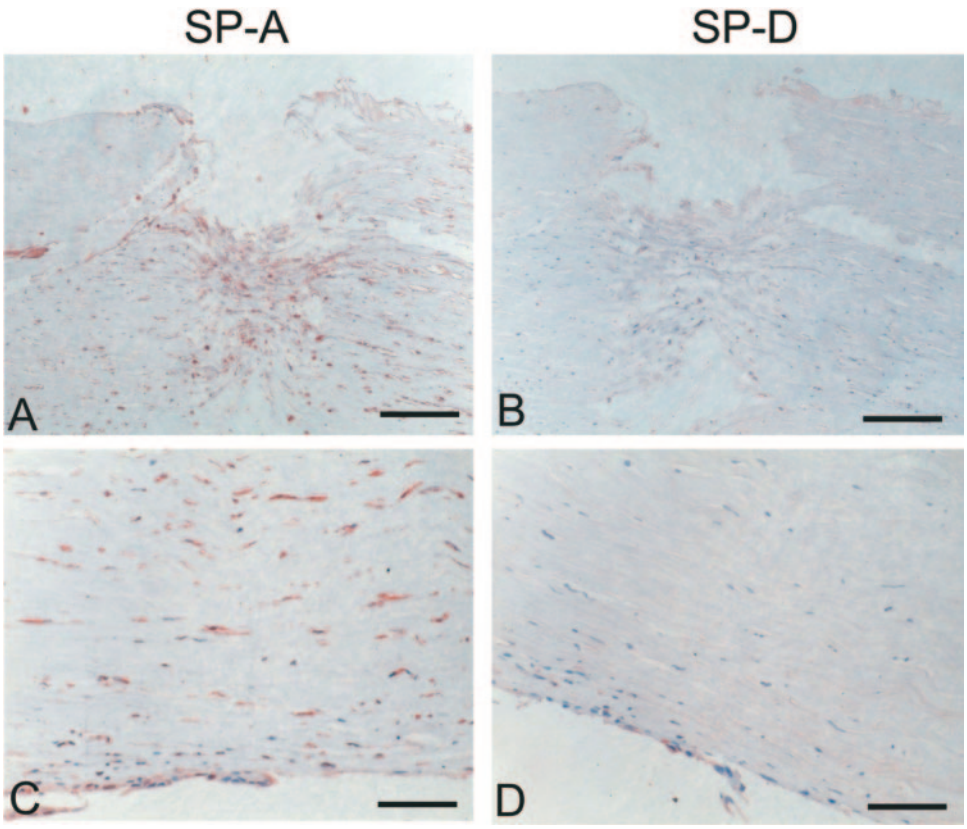

FIGURE 8. Corneal ulceration after infection with *S. aureus* (bacterial ulcer) incubation with specific antibodies against SP-A and SP-D. Cells surrounding the area of the ulcer showed positive reactivity (*red*) (**A**, **B**). Stromal and endothelial cells also stained positive (**C**, **D**). (**A**–**D**) Hemalum. Scale bars: 112 μm (**A**, **B**); 56 μm (**C**, **D**).

already been demonstrated for several mucosal surfaces,[30] including mouse, porcine, and human lacrimal glands[31,32,42] and human tear fluid.[33] Interestingly, the results of Ni et al.[33] revealed SP-D reactivity throughout the mouse corneal epithelium, whereas our results in human cornea showed staining only for the most superficial layer of the corneal epithelium cells. This result indicates species-specific and tissue-specific expression of the surfactant proteins for healthy human cornea, whereas in pathologic samples, a similar distribution pattern of the proteins was observed compared with mouse. Furthermore, because of the variable postmortem time frame of the cadavers, partial degradation of the surfactant proteins in the cornea might be possible, leading to loss of reactivity.

Once again, with regard to SP-A, our results indicated species-specific differences. For example, human SP-A and SP-D seemed to act in a synergistic manner; proteins were present in all tissues of the lacrimal apparatus and the ocular surface. By contrast, in mice, or at least in C57/BL6 mice, SP-A protein appeared to be predominantly expressed in the lung and seemed to be absent in the lacrimal apparatus.[32] Thus, mice overexpressing [43] or lacking SP-D[26,28] and mice overexpressing SP-A [44] will be useful models in which to explore the role of the proteins in host defense of the tear system, whereas mice lacking SP-A will not be as useful for studies of SP-A in the tear system.

No immunohistochemical reaction was observed in endothelial or stromal cells of healthy cornea. By contrast, analysis of corneal abnormalities (*S. aureus*–induced corneal ulceration and herpetic keratitis) revealed the presence of SP-A and SP-D in corneal endothelial cells, in cells of the corneal stroma, and in corneal epithelium, suggesting that the production of both proteins is inducible or upregulated in cornea, as was recently demonstrated in human prostate.[45]

In addition, in keratoconus, SP-A and SP-D were detected in all layers of the cornea. Keratoconus, a noninflammatory disease resembling a corneal thinning disorder, includes the development of a localized conical protrusion. Histologically, fine anterior scars are visible that are caused by idiopathic breaks in Bowman membrane. The Descemet membrane also shows signs of disruption that may lead to destruction of the integrity of the corneal endothelial barrier and to stromal edema. All processes lead to destruction of the corneal structure. Therefore, protection mechanisms such as the induction of SP-A and SP-D may be useful. Such protection in the form of antiapoptotic or motogenic effects was shown for trefoil factor family peptide 3 in keratoconus.[46]

Cultured immortalized HCE and HCjE cells revealed constitutive production of SP-A and SP-D before stimulation. After stimulation with different cytokines and bacterial components, the staining intensity of the cells increased subjectively. However, we did not quantify the staining intensity or the concentration of either protein in the culture supernatants. These will be interesting to determine in future investigations.

Based on our present findings concerning the subjectively enhanced expression of the surfactant-associated proteins in different pathologic samples and stimulated cell lines, we hypothesized a contribution of SP-A and SP-D to the immune system of the ocular surface and the tear film. In this context, Ni et al.[33] showed that the use of recombinant human or mouse SP-D reduces the invasion of corneal epithelial cells by *Pseudomonas aeruginosa*. Positive reactivity for SP-A and SP-D in defense cells (which were not characterized in the present study), visible in some of our samples, are a link to further functions of both collectins. It has been demonstrated in this context that SP-A and SP-D strongly enhance and facilitate phagocytosis of several pathogens by macrophages.[45]

Binding and opsonizing infectious pathogens and binding to the surfaces of host defense cells to promote or inhibit immune cell activity through multiple cellular pathways are important not only for the ocular surface but also for the mucous membrane of the nasolacrimal passage to protect against dacryocystitis, the most frequent disease of the efferent lacrimal system,

because most pathogens coming from the ocular surface are drained through the efferent tear ducts. Therefore, SP-A and SP-D might act in concert with several other antimicrobial substances of the innate immune system.[47]

In addition to protection of the nasolacrimal ducts, the cornea and, ultimately, the whole eye against invasion of pathogens, elucidation of further functions of SP-A and SP-D at the ocular surface and in the tear film will be of considerable interest. Thus, SP-D has just recently been demonstrated in lung allergy to show anti-inflammatory action by regulating chemotaxis and degranulation of human eosinophils.[48] SP-A is able to bind pollen from *Populus nigra* in a calcium- and sugar-dependent manner,[49] thereby directly interacting with allergens. SP-A and SP-D have also been shown to contribute to the physical structures of lipids in the alveoli and to the regulation of surfactant function and metabolism.[50] Sequentially targeted deficiency of SP-A and SP-D leads to progressive alveolar lipoproteinosis, lung emphysema, hyperplasia and hypertrophy of type 2 alveolar epithelial cells, as well as intracellular surfactant accumulation.[51, 52]

In conclusion, our data permit deeper insight into the surfactant system of the human lacrimal apparatus, ocular surface, and tear film. As a consequence of their physiologic functions, SP-A and SP-D–dependent pathways are targets for clinical therapies designed to limit the proliferation of microorganisms and to ameliorate inflammation after ocular surface and efferent tear duct infection.

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