Tear Film and Ocular Surface Surfactants

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Abstract: The hydrophobic surfactant proteins B (SP-B) and C (SP-C) are tightly bound to phospholipids. These proteins play important roles in maintaining the surface tension-lowering properties of pulmonary surfactant. Surfactant protein A (SP-A) and D (SP-D) are extremely hydrophilic and are thought to have a role in recycling surfactant and especially in improving host defense in the lung. Moreover, SP-A supports the hydrophobic surfactant proteins during surfactant subtype assembly and inhibits secretion of lamellar bodies into the alveolar space. During recent years surfactant proteins have also been detected at locations outside the lung such as the lacrimal apparatus. In this review, the latest information regarding SP function and regulation in the human lacrimal system, the tear film and the ocular surface is summarized with regard to mucous epithelial integrity, rheological and antimicrobial properties of the tear film, tear outflow, certain disease states and possible therapeutic perspectives.

Keywords: Surfactant proteins, ocular surface, tear film, lacrimal apparatus.

INTRODUCTION

Each year worldwide 55 million ocular injuries occur (WHO-Program for the prevention of blindness). Ocular trauma and corneal ulcerations due to infection are the most significant causes of corneal blindness yearly leading to 1.6 million new instances of bilateral blindness, 2.3 million instances of serious limitation in visual acuity, and 19 million instances of unilateral blindness. In Germany, approximately 30 million people wear glasses or contact lenses. In the USA, the number approaches 120 million. In 1995, the number of radial keratotomies (RK) reached 250,000 in the USA (National Eye Institute). Meanwhile, RK has been almost completely superseded by LASIK (laser assisted in situ keratomelieusis) (1.55 million operations in the USA in the year 2000). These numbers have increased appreciably over the last seven years. The laser market has a yearly growth rate of 10-25%. Although LASIK is a great deal safer and associated with fewer complications than RK and photoreactive keratoectomy, standardized numbers on the occurrence of LASIK related complications have not yet become available. To date, the only relevant publications have been based on small case numbers at individual centers.

Due to the difficulties involved in treating corneal blindness once it has developed, it is, from a medical point of view, well worth the effort to search for therapeutic strategies that could improve healing early and effectively, reduce infections and diminish corneal scarring in order to reduce the number of instances of persistent corneal blindness.

In ophthalmological practice, bacterial keratitis and conjunctivitis are among the most frequently seen problems. They represent an important component of numerous infections of the eye, particularly in patients after penetrating corneal injury, after lengthy periods of contact lens wear, after refractive corneal surgery or under immune suppression therapy [1-4]. The most frequent causal agents are the gram positive pathogen *Staphylococcus aureus* and the gram negative bacteria *Pseudomonas aeruginosa* [5, 6], but also viruses, e.g. the *Herpes simplex* virus, can instigate keratitis. Bacterial by-products and toxins as well as the host's inflammatory reaction often bring about extensive tissue damage with persistent scarring or even loss of sight [1].

It has long been known that tear fluid contains various anti-bacterially active substances. These substances are synthesized by various cells found in the lacrimal glands, the accessory lacrimal glands, the conjunctiva and the cornea. Of these antimicrobial substances, lysozyme [7], mainly effective against gram positive bacteria, has been most extensively described and studied. Further substances include the enzyme β-Lysin, which destroys bacterial cell membranes working synergistically with lysozyme [8], the iron-binding protein Lactoferrin, which inhibits growth of *Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* [9, 10], as well as complement factors of the alternate pathway, which are activated directly by microbial products, e.g. endotoxins [11]. In the past few years, the research group surrounding Bernhard Redl has been able to better characterize the protein lipocalin present in the lacrimal fluid and has recently demonstrated that tear-specific lipocalin is bacteriostatic to *Eschericha coli* and various fungi *via* iron deprivation [12]. Recent research has further shown that lacrimal fluid contains secretory phospholipase A_2, an enzyme which, along with lysozyme and ß-lysin is particularly effective against gram-positive bacteria [13, 14].

A further mechanism of defence is represented by antimicrobial peptides. It is assumed that some of these peptides are able to kill microorganisms directly *via* formation of pores. For the remainder of these peptides the mechanism of activity has not yet been fully elucidated. The best-characterized group of antimicrobial peptides are the human

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defensins that can be subdivided into two groups, α-defensins and β-defensins. Further antimicrobial peptides that have been described on the ocular surface include BPI (bactericidal permeability-increasing protein), CAP37 (heparin-binding protein), LL37 (human cationic antimicrobial protein/hCAP-18), LEAP1 (liver expressed antimicrobial peptide 1 or heptcidin) and LEAP 2 and angiogenin. A current review of antimicrobial pepitides at the ocular surface has been presented by McDermott [15].

SURFACTANT PROTEINS

The surfactant-associated proteins A and D (SP-A and SP-D) have been extensively described in research related to the lung. Immunological functions in both the non-specific and specific immune defence systems have been ascribed to them. SP-A and SP-D are representatives of the collectin-family of the C-type, among with numerous other molecules of known immunological function may be counted.

According to the current understanding of the mechanism of C-type collectins, exposed microbiological carbohydrates (on the surface of diverse microorganisms) bind to a carbohydrate recognition domain inherent to the proteins. In this manner, opsonisation and acceleration of the defence reaction to the microorganism is achieved.

SP-A is a 28-36 kDa soluble protein first recognized in type II pneumocytes of the pulmonary epithelium and is expressed by these cells. SP-A isolated from broncheoalveolar lavage specimens is mainly present in the form of surfactant-lipid-aggregates. Furthermore, the presence of SP-A augments the reduction in surface tension induced by SP-B in pulmonary alveoli. Nonetheless, deletion of the SP-A gene in mice does not lead to a decline in lung stability, albeit the protein inhibition of surfactant reveals an important functional relevance of SP-A [16]. SP-A bind to type II pneumocytes and to immune cells as well as to some extent to macrophages [17]. Beyond this, it has been demonstrated that SP-A deficient mice exhibit a weakened defence to various pulmonary pathogens [18].

SP-D is a 43 kDa protein synthesized not only by type II pneumocyte epithelium, but also by various cells of the respiratory tract and mucous cells of the stomach. It is characterized by a very high structural similarity and, correspondingly, homology to SP-A and other mammalian specific lectins [19]. The lectin domain of SP-D, mediated by calcium ions, bind to carbohydrates and lipids, playing a role in the innate immune defence particularly against bacterial, viral and fungiform pathogens. It interacts directly with a number of microorganisms such as influenza virus A [20], *Pseudomonas aeruginosa* and *Escherichia coli* [21, 22]. In addition, its important role in lipid homeostasis of the lung could be demonstrated through the selective deletion of SP-D in mice [23]. SP-D deficient mice develop alveolar lipidosis, which is accompanied by the activation of macrophages and an increased activation of metalloproteinases [24]. Beyond this, the SP-D deficient mice develop emphysema-like pathological changes reflecting the critical role of the surfactant proteins in the regulation of pulmonary inflammation.

The first description of proteins in organic extracts of extracellular surfactant and lamellar bodies was presented by Phizackerley *et al*. in 1979 [25]. The characterization and purification of these proteins proved quite difficult due to their high hydrophobicity and low molecular weights. Finally, it could be demonstrated that pulmonary surfactant contains at least two different small molecular weight hydrophobic proteins, known as SP-B and SP-C [26, 27]. Using non-reducing preparation-conditions the molecular weights of SP-B by means of SDS-PAGE have been estimated at approximately 15-18 kDa, whereas under reducing conditions a suitably separable product of approximately 7 kDa was obtained [28, 29]. SP-B, post-translationally produced from a preform is also able to form oligomeres of various sizes *via* disulfide bridges [29]. SP-C, utilising 33-35 amino acids, is one of the smallest and at the same time most hydrophobic proteins known, with a molecular weight of 4-6 kDa [30, 31]. Its primary translation product consists of 191 amino acids. As does SP-B, SP-C also undergoes extensive post-translational modification, for example, glycosylation, acylation or esterification with fatty acids [32-35]. In contrast to the sugar binding collectin-like SP-A and SP-D, SP-B and SP-C are decisive in the formation and stability of surface active layers (membranes) and beyond this are a prerequisite for the absorption of phospholipids at the air-fluid boundary [36, 37]. Due to their hydrophobic properties, SP-B and SP-C are of great pathophysiological relevance in acute respiratory distress syndrome (ARDS) as diverse plasma proteins inhibit the formation of pulmonary surfactants and thus decrease alveolar surface tension [38]. In this context it has been reported that the deletion of SP-B in newborn rabbits leads to serious disturbance of the affected surfactant and injury to the alveolar surface of therewith engendered respiratory stress in the rabbit [39]. The function of SP-C is comparable to SP-B in that it is characterized by its direct influence and interaction with biological and phase interfaces; it namely reduces surface tension and increases the ability to absorb the surfactant, like an anchor mediating between the phopholipid layer and the aqueous phase.

SURFACTANT PROTEINS OF THE TEAR FILM, OCULAR SURFACE AND LACRIMAL APPARATUS

The Meibomian glands of the eyelids are responsible for the production of the superficial lipid components of the tear film which counteract evaporative tendencies of the deeper tear film layers directly adjacent to and protecting the ocular surface [40]. The lacrimal glands, along with the accessory glands of the lids, express the watery components of the tear film. These play a role not only in moistening of the ocular surface, but also in defending against potentially pathogenic microorganisms [13]. The mucous components of the tear film consist for the most part of secretory mucins produced in conjunctival goblet cells, the lacrimal and accessory lacrimal glands, as well as of membrane bound mucins synthesized by conjunctival and corneal epithelial cells [41-45]. Membrane bound mucins may be split from the epithelial surface *via* a process known as shedding, and thus may also enter the aqueous component of the tear film [44, 45].

For a long time it has been supposed that superficially active substances, similar to the surfactant system of the lung, are of importance not only in tear film but also in the auditory tube and on the skin [46]. Various studies have documented the presence of surfactant-associated proteins A, B and D in the auditory tube [47] and of SP-A, B, C and D in the skin [48]. The presence of SP-D has already been described in tear film and lacrimal glands [49-51]. Ni *et al*.

[52] was able to show that beyond this, SP-D is present in the cornea of mice and has protective effects against keratitis caused by *P. aeruginosa*. Although Dobbie *et al*. [53] had already presented immunohistochemical evidence of the presence of SP-A in the human lacrimal gland, these findings were pushed into the background by Stahlmann *et al*. [50] and Ni *et al*. [52] as the latter authors could not demonstrate SP-A in tear fluid of mice and their findings were taken to be conclusive for humans as well. Our group has now been able to show that not only SP-D, but also the surfactant-associated protein A, along with SP-B and SP-C is present at the ocular surface, in the lacrimal apparatus and in tears [54, 55]. Thereby, all of the surfactant-associated proteins (SP's) proved to be detectable at the mRNA and protein levels throughout the lacrimal apparatus and on the ocular surface in all tissues studied (lacrimal glands, conjunctiva, cornea, lacrimal ducts) as well as in tear fluid itself. Interestingly, SP-A and SP-C in tear fluid, and SP-C in all examined tissues, show an expression pattern differing from that of lung surfactant proteins. This is probably due to tissue specific post-translational or post-transcriptional modifications of the proteins and may lead to differences in the spectrum of activity of the surfactant proteins. SP-A, -B, -C and –D are found in the acinar cells of the lacrimal gland and the accessory tear ducts, in the conjunctival epithelial cells and in columnar epithelial cells (particularly apically) as well as in serous portions of seromucous glands in the tear ducts and the accessory tear ducts. Goblet cells do not produce any of the four SP's. In contrast to tear fluid, the aqueous humor does not show the presence of SP's under physiological conditions [54]. In healthy corneal specimens, the two collectins SP-A and SP-D are only found as a thin film in the apical region of the superficial cells. SP-B and SP-C are immunohistochemically undetectable within and on the surface of the cornea [55]. In contrast, pathologically altered corneae show a completely different distribution pattern. In association with herpes keratitis and corneal ulceration (caused by *S. aureus),* SP-A and SP–D are found in the vicinity of the lesion sites as well as in invading immune cells; in the corneae of patients with keratoconus the entire epithelium and the endothelium react positively. Cultured corneal and conjunctival epithelial cell lines also produce SP-A and SP-D [54]. Stimulation of corneal and conjunctival epithelial cell lines [56, 57] with various cytokines and bacterial supernatants lead to an increase of both collectins. Thus, in addition to SP-D which has already been reported to be present in tear film [49, 50, 52], there are also SP-A, SP-B and SP-C proteins in the lacrimal fluid and on the ocular surface.

CONCLUSIONS

Based on the present status of knowledge including that gleaned from animal experimental studies on SP-D [52, 58] it can be assumed that the collectins, SP-A and SP-D, are involved in pathological processes at the ocular surface and function here, as well as in the efferent tear ducts, in the service of non-specific natural immune defence and in the activation of the adaptive immune system. As a substance intrinsic to tears they protect the ocular surface in conjunction with immunoglobulin A (IgA), defensins and mucins against infection by *P. aeruginosa*, *S. aureus* and other pathogenic microbes [15, 52, 59-62]. In the efferent tear ducts they are also active in conjunction with IgA, antimicrobial peptides and mucins in preventing the formation of dacryocystitis [54, 55, 63-70].

In view of the fact that the hydrophobic surfactant proteins SP-B and SP-C have an expanding influence on the surface tension of the air-liquid interface on top of the alveolar lining layer, a similar effect could be discussed in relation to the tear film and the tear fluid at the human ocular surface and efferent tear ducts. In this context we provide a completed and modified scheme of the tear film which now contains the evidenced and investigated surfactant proteins with respect to their physicochemical properties and discussed putative functions in tears, the lacrimal apparatus and at the ocular surface (Fig. **1**). This view demonstrates the small hydrophobic surfactant proteins B and C embedded into the lipid component of the tear film, orientated regarding to their amphiphilic character. Furthermore, the water-soluble and polymerizeable collectin-like surfactant proteins A and D are arranged within the aqueous component of the tear film along with the already known different secreted and shedded mucins. This hypothetical model supports possible functions of surfactant proteins with regard to severe pathologies of the ocular surface e.g. dry eye syndrome and bacterial or viral infections. Dysfunctions of the complex ocular surfactant system would probably lead to considerable visual impairment and disturbances of the immune defence system of the ocular surface and lacrimal apparatus. Hypothetically, absence of the small hydrophobic surfactant proteins B and C could result in alterations of tear film stability and as a consequence in interruption of the tear film itself leading to symptoms of dry eye syndrome such as for example dry spots. Disturbed production or lack of the immunologically important surfactant proteins A and D could lead to impairment of innate and adaptive immunity at the ocular surface and in the lacrimal system resulting in various bacterial and/or viral infections.

PERSPECTIVES

By means of the developement of a gene construct for all four surfactant proteins, which includes open reading frames for mature and preforms of the proteins, a prerequisite has been formed for the establishment of a system of expression *via* which all four SP's can be manufactured using recombinant methods. The thus manufactured recombinant SP's could serve in functional studies on questions pertaining to the ocular surface and the lacrimal system. Some recombinant SP's have already been manufactured (for a review see [71, 72]). With the software programs and methods available today, it is possible to create a reliable model of the 3D-structure of proteins and to identify the active sites by using comparative protein modelling or threading. Blocking or modification of putative active sites could alter the functionality of the proteins and lead to completely new proteins at a functional level. Up to now there is no information on the ocular surface and lacrimal system of surfactant-deficient mice, which are already available for all four SP's [23, 73-75]. In studies on conditions of chronic infection of the ocular surface such as that found, for example, in dry eyes, it would be of interest to know whether individual SP formation is increased or decreased. An increase in surfactant formation over a lengthy period of time would very possibly have negative consequences for the body as a whole. In this context it has been demonstrated that SP-D exhibits proat-

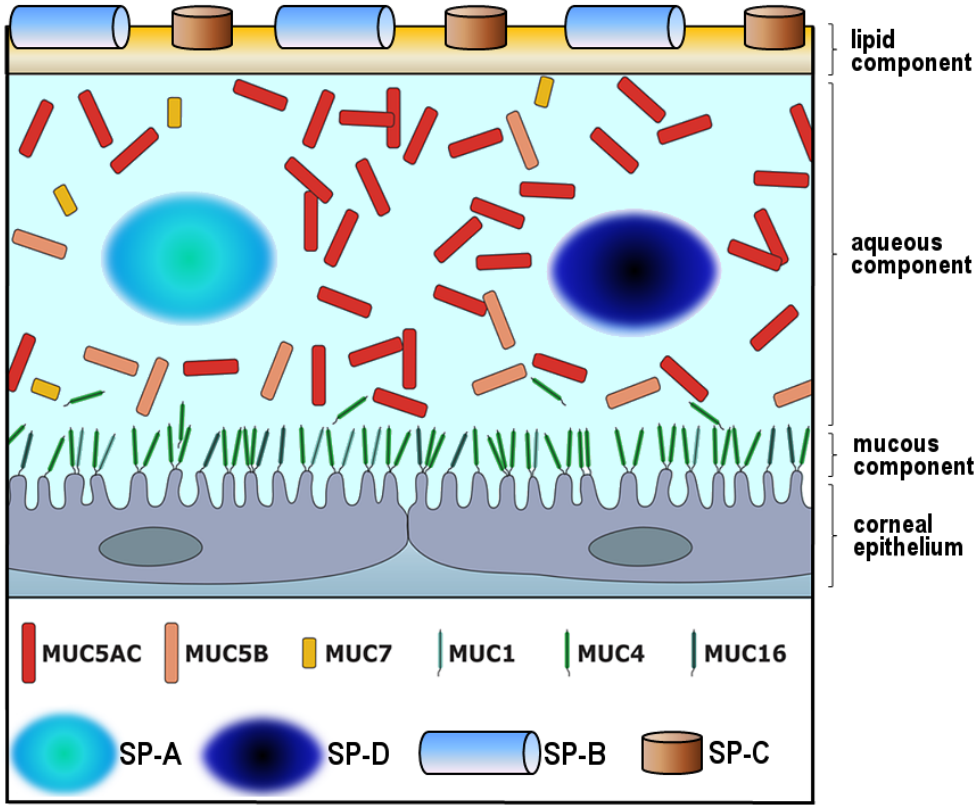

Fig. (1). The figure demonstrates a modified and completed scheme of the present understanding of the tear film in accordance to that recently published by Paulsen (2006). It displays the arrangement of the water-soluble and putative immunologically important surfactant proteins A and D within the aqueous phase of the tear film. Furthermore, the small hydrophobic surfactant proteins B and C are illustrated. They are embedded into the lipid-phase of the tear film with respect to their physicochemical propteries. Moreover, most of the already known and still investigated mucins of the ocular surface and tear film are shown along with SP-A and SP-D.

herogenous activity in mice [76]. Studies on the surfactant-protein system of the ocular surface have just begun and lead us to expect interesting new discoveries in regard to future therapeutic perspectives.

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LIST OF ABBREVIATIONS

SP	=	surfactant protein
WHO	=	Word Health Organization
RK	=	radial keratotomy
LASIK	=	laser assisted in situ keratomelieusis
CAP37	=	heparin-binding protein
LL37	=	human cationic antimicrobial protein
hCAP18	=	human cationic antimicrobial protein
LEAP1	=	liver expressed antimicrobial peptide 1/heptcidin
LEAP2	=	liver expressed antimicrobial peptide 2
SDS-PAGE	=	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
ARDS	=	acute respiratory distress syndrome
mRNA	=	messenger ribonucleic acid
IgA	=	immunoglobulin A

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