Stony Brook University

Involvement of Surfactant Proteins in Ocular Innate Defense

A Dissertation Presented

by

Xiaolan Lin

to

The Graduate School

in Partial Fulfillment of the

Requirements

for the Degree of

Doctor of Philosophy

in

Materials Science and Engineering

Stony Brook University

May 2010

Stony Brook University

The Graduate School

Xiaolan Lin

We, the dissertation committee for the above candidate for the

Doctor of Philosophy degree, hereby recommend

acceptance of this dissertation.

Miriam Rafailovich- Dissertation Advisor
Professor, Materials Science and Engineering

Gary Halada- Chairperson of Defense
Associate Professor, Materials Science and Engineering

Yizhi Meng
Assistant Professor, Materials Science and Engineering

Richard Clark
Professor, Biomedical Engineering

This dissertation is accepted by the Graduate School

Lawrence Martin
Dean of the Graduate School

Abstract of the Dissertation

Involvement of Surfactant Proteins in Ocular Innate Defense

by

Xiaolan Lin

Doctor of Philosophy

in

Materials Science and Engineering

Stony Brook University

2010

The ocular surface is a delicate structure that susceptible to injury and infection. Ulcerative keratitis is a painful eye condition characterized by stromal infection, ulcerative epithelium, decreased vision and cornea scar. Due to the reason that formation of vascularization, fibrosis or scarring in corneal epithelium can cause disastrous effects on vision, inflammatory response in the eye must be tightly controlled in space and time. Ocular innate defense system consists of antimicrobial peptides and immune cells that secreted or accumulated at the surface; they work in concert to eliminate intruding pathogens. Under normal circumstances, innate immunity suffices in providing quick, non-specific protection against infection. As opposed to adaptive immunity which usually takes longer and thus has more destructive effects on ocular tissues, innate immunity is more benign as its occurrence is restricted to superficial tissues such as eyelid, limbus and conjunctiva, of which the blood supplies bring immune cells to the inflamed sites for clearance of microbial and apoptotic cell debris.

Surface-tension associated proteins (SP) that present in corneal epithelium and tear fluid has emerged as the new player in innate immunity of the eye. In this study, function of SP protein in corneal innate immune modulation was evaluated by using a human corneal epithelial cell line. Cells were depleted of SP by transfecting with short interfering RNA and stimulated cytokine production was determined. Conversely, exogeneous SP was found to reverse the impaired immune response in SP-depleted cells in a dose-dependent manner. This study is the first to demonstrate the role SP as immune-regulator in corneal epithelium. Combined with their known activity in direct killing of bacteria, SP may have therapeutic implications in ocular infectious diseases.

Table of Contents

List of Figures

Abbreviations

AFM	Atomic force microscopy
ELISA	Enzyme-linked immunosorbent assay
HCE-2	Human corneal epithelial cell line
IL-8	Interleukin-8
LPS	Lipopolysaccharide
nNHEK	Normal neonatal human epidermal keratinocytes
PCR	Polymerase Chain Reaction
PGN	Peptidoglycan
rhSP	Recombinant human surfactant proteins
S.aureus	Staphylococcus aureus
siRNA	Short interfering RNA
SP	Surfactant proteins
TNF-α	Tumor necrosis factor-α
UV	Ultraviolet

Acknowledgements

I am most indebted to my advisor, Professor Miriam Rafailovich, for the opportunity to do my dissertation under her guidance. I wish to thank her for her patience and belief in this work and in me. She gave me the precious guidance I needed; in the mean time she allowed me to freely pursue my ideas. Her wisdom and kindness are inspiring, not only to my research work, but also to my daily living.

I wish to thank deeply to Professor Richard Clark, Yizhi Meng, Ph.D., and Gary Halada, Ph.D. for their candid advice and unconditional support during the time the research was undertaken, in spite of their own busy schedules. Their enormous knowledge about biology, chemistry, and material science has greatly helped this project to succeed.

Above all, I wish to thank my loving parents, Junying Fan and Wenqing Lin, who have believed in me and supported me without ever asking for anything. In the past year I should have spent more time with them.

I thank my beloved husband, Zhen Liu. Without him, I probably would have fixed to the chair in the office during the last months of this project. I am deeply grateful for his positive attitude and encouragement, and most of all, for his love and caring.

January 2010

Xiaolan Lin

1 Introduction

The ocular surface is covered by a stratified, squamous and non-keratinizing epithelium that is about 5-7 cells thick (Knop et al. 2007). Corneal epithelium opens to the environment, and thus is exposed to a vast variety of biological, chemical or mechanical insults. To maintain its proper barrier functions, healthy corneal epithelium along with the tear fluid it bathed in, forms an intricate defense system ranging from simple rinsing by tear fluid to complex innate or adaptive immunity. Ultraviolet irradiation (UV) and pathogen invasion represent the most common environment threats to the eye. The ocular epithelium as the most outer layer of the eye absorbs a substantial amount of UV-B irradiation than any other ocular tissues, therefore acting as a filter to protect lens and retina from UV induced damages (Ringvold. 1998, Kolozsuari et al. 2002). Exposing to high doses of UV-B however, would cause cell shedding to the tear pool (Shimmura et al. 2004). Small amount of cell debris can be simply removed by mechanical rinsing by tear fluid; but significant cell death in the ocular surface may increase the susceptibility of the eye to pathogen invasions.

In addition to UV irradiation, the ocular surface also confronts deleterious pathogens (bacteria, virus, and fungus) on a daily basis. Cells' innate immunity serves as the first line defense against invading pathogens and involves such components as antimicrobial peptides (e.g. β-defensin) and phagocytes (e.g. neutrophils, macrophages) for quick and non-specific clearance of dead cells and foreign objects. Under normal circumstances, innate defense mechanism is sufficient for protecting the eye from infection. However, in severe cases adaptive immune responses are activated to summon immunologically responsive cells (e.g. T-cells and B-cells) to tackle the challenge. This process takes relatively longer

and has more profound consequences. Excess and prolonged inflammatory process could generate undesirable outcomes including deep wounds and scar tissue formation. Ocular keratitis is an eye condition in which chronic inflammation leaves behind scar tissues in the corneal surface, causing blurry vision or even blindness. Therefore, quick onset and resolution of an inflammation is vital not only for a timely recovery of ocular function, but also for scar-less healing essential for maintaining corneal transparency and visual acuity.

Inflammatory reaction is an orchestrated process mediated by a myriad of proinflammatory cytokines such as IL-1a, IL-1b and TNF-α that subsequently induce the expression of chemokines such as IL-8 in keratinocytes and epithelial cells (Wang et al. 2001, Nakamura et al. 2002, Azghani et al. 2002, Sun et al. 2006, Venza et al. 2007). IL-8, now referred to as CXC chemokine ligand 8 (CXCL8) is one of the most potent neutrophil chemoattractants (Barker et al. 1991). In the case of corneal inflammation, early augmentation of IL-8 may contribute to timely recruitment of neutrophils to the avascular cornea (Elner et al. 1991, Cubitt et al. 1993). Additionally, IL-8 is shown to stimulate α-smooth muscle actin production in fibroblasts and caused the wounds to contract and close more rapidly (Feugate et al. 2002). IL-8 is also chemotactic for fibroblasts, accelerates their migration and deposition of extracellular matrix proteins during wound healing (Kuhlmann et al. 2009). Unlike skin, inflammation induced vascularization, fibrosis and scarring can have disastrous effect on eye function. Corneal keratinocytes and epithelial cells are able to produce IL-8. Secretion of IL-8 at the ocular surface would induce local leukocyte recruiting from superficial tissues like

conjunctiva and eyelids; this could minimize deep-tissue inflammation and help to prevent tissue damage and vision loss.

Surfactant proteins (SP) were originally discovered in alveolar lining of the lung (Phizackerley et al. 1979). In the past several decades, most studies regarding the functions of SPs have been focused on their potential roles in the lung. Identification of surfactant proteins (hydrophilic SP-A and SP-D, hydrophobic SP-B and SP-C) in corneal surface and tear fluid must be attributed to Brauer and his colleagues (Brauer et al. 2007). Considering that epithelium of lung and corneal share many common features, researches of SP in corneal may greatly benefit from lung research conducted in the past. However corneal epithelium has its unique properties and functions; at present time SP functions in the corneal surface remain more of a speculation if not a mystery. In lung alveolar, SP-A and SP-D were known to primarily mediate host defense by binding to intruding pathogens, whereas SP-B and SP-C maintain low surface tension of a phospholipid monolayer by facilitating the adsorption and spreading of phospholipids at the air/ liquid interface (Ding et al. 2001). As known from pulmonary surfactant system, SP-B and SP-C are speculated to be an integral part of the corneal epithelium and may serve essential role in maintaining a stable and continuous layer of tear fluid.

Even though SP-A and SP-D have long been acknowledged for their active roles in host defense, opinions are divided on how they mediate an inflammatory reaction. Quite a few studies supported the view that they are proinflammatory (Meloni et al. 2002, Blau et al. 1994, Kremlev et al. 1994, Kremlev et al. 1997), some believed they are anti-inflammatory (Synder et al. 2008, Borron et al. 2000), and many recent

studies believed in both (Gold et al. 2004, Vaandrager et al. 2000, Wright. 2005, Takahashi et al. 2006, Kingma et al. 2006, Chaby et al. 2005, Haczku. 2008). When it comes to SP-B and SP-C, much less is known for their involvement in immune responses, possibly due to the technical difficulties in characterizing and purifying these small and hydrophobic proteins. A working hypothesis is that all types of SP work in concert with cytokines in response to a harmful insult. This study sets out to shed the light on the role of SPs in immune responses, specifically IL-8 production of corneal epithelium triggered by UV-B irradiation or peptidoglycan (PGN) (cell wall components from gram-positive bacteria). This is the first study to explore the functions of SPs in an induced inflammatory reaction in corneal epithelial cells.

Additionally, feasibility of incorporating SP in ophthalmic biomaterials such as silicone lenses and artificial tear fluids will be evaluated. The underlying hypothesis is that wettability of silicone-based biomaterials may be improved by surface adsorption of SP. Further, SP may have the potential to replace surfactants currently used in an artificial tear formulation.

2 Review of the literature

2.1 Structures of surfactant proteins

Surfactant proteins (SP) are synthesized by differentiating epidermal keratinocytes. Their primary translation products are translocated into the lumen of endoplasmic reticulum (ER) with the aid of N-terminal propeptide. After translocation into the ER, SPs are transported to the Golgi and from here to lamellar bodies that contain SPs and lipids. Mature SPs are secreted to the intracellular spaces after keratinocytes reach terminal differentiation. SPs are presented at the air/liquid interface of the monolayer. There are four types of SP, hydrophilic SP-A and SP-D and hydrophobic SP-B and SP-C.

2.1.1. SP-A and SP-D

SP-A and SP-D are structurally related and belong to a subgroup of mammalian lectins called “collectins” (or C-type lectins, group III). Each collectin monomer contains four distinct domains: 1) a carbohydrate recognition domain (CRD) (or a lectin domain); 2) a neck domain containing a short hydrophobic stretch of amino acids and an amphipathic helix; 3) a collagen like domain consisting of Gly-X-Y repeats; 4) an amino terminus containing a cysteine involved in interchain disulfide bond formation. Basic structural units consist of three monomers oriented in parallel with their collagen-like tails folded into triple helix. In mature SP-A, six of these trimers are bound together with their CRDs pointing in one direction and their collagen tails in the other, resembling a bunch of tulips. SP-D consists of four of these basic trimer units and positioned tail-to-tail in a cross-like form (Figure 1).

SP-A is a 28-36 kDa hydrophilic collagenous glycoprotein encoded by two genes (SP-A1 and SP-A2) in human (Ketyal et al. 1992). SP-D is a 43

kDa hydrophilic glycoprotein. The collagen domain of SP-D is much longer than that of SP-A (59 vs 24 Gly-X-Y repeats) and it has no kink in the triple helix, therefore SP-D has a much longer collagen triple helix than SP-A. CRD of SP-D primary binds to sugar; whereas CRD of SP-A binds not just to sugar, it can also engage in protein-protein and protein-lipid interactions. Structural differences of SP-A and SP-D have in many cases attributed to their functional differences. SP-A is associated with surfactant lipids in lung lavage, whereas most SP-D is not. Interactions of their CRDs with pathogens are also different. For example, when interact with lipopolysaccharide (LPS, endotoxin), CRD of SP-A binds to the protein and lipid components of LPS; whereas CRD of SP-D binds to the polysaccharides of LPS (Tenner. 1999). When binds to influenza virus, SP-A binds to viral neuramidase via its sialic acid residues present on the CRD, whereas CRD of SP-D binds to glycoproteins in the virus membrane (Haagsman. 1998).

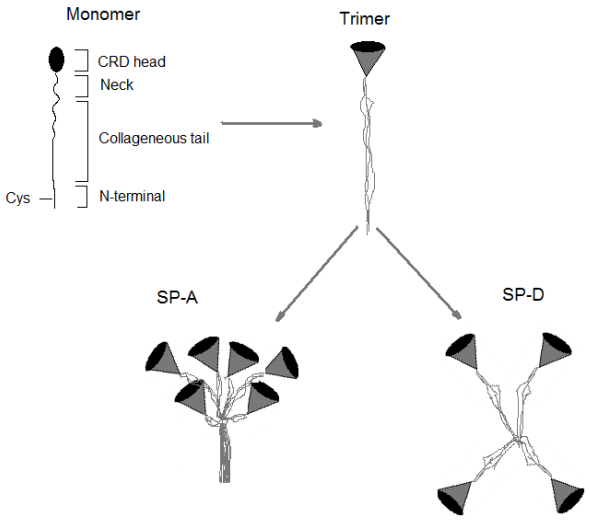


Fig.1 Structures of SP-A and SP-D

2.1.2 SP-B and SP-C

SP-B and SP-C are small hydrophobic proteins (8 kDa and 4 kDa) that are functionally related. They are encoded on chromosome 2 and 8 respectively. Primary translational product of SP-B has 381 amino acids (a.a), containing a 23 a.a N-terminal signal sequence. Cleavage of the signal sequence yields a proprotein of 358 a.a. that consists of the mature protein (a.a 201-279) flanked by an N-termial propeptide (a.a 24-200) and a C-terminal propeptide (a.a 280-381). Cleavages of the propeptides release the mature form of SP-B. Although the two terminal flanking arms have an anionic net charge (-4 and -2, respectively), the charge of mature SP-B is highly cationic (+7). Like SP-B, SP-C proprotein contains two flanking arms at N-terminal (a.a 1-23) and C-terminal (a.a 59-179). The amino acids of the mature SP-C (a.a 32-57) form α-helix capable of spanning a membrane bilayer. This α-helix is made up of highly hydrophobic amino acids, mostly valines and leucines. The minor part of SP-C that not spanning a membrane is hydrophobic as well.

NMR spectroscopy revealed the secondary and tertiary structures of both proteins. SP-B consists up to 45% α-helical structures and at least 20% β-sheet; whereas SP-C is composed predominately of α-helical loops although β-sheet structures were observed at higher concentrations.

SP-B is a member of the saposin-like family of proteins (SAPLIP). Several SAPLIP members (e.g., NK-lysin, granulysin and amoebapore A) kill bacteria by increasing permeability of bacterial membranes but their mechanisms differ. For example, NK-lysin and granulysin contain positively charged amino acids on the surface which allow them to interact with negatively charged bacteria resulting in bacterial membrane

destabilization and/or permeabilization (Bruhn et al. 2003, Ernst et al. 2000). Amoebapore A is much more hydrophobic and permeabilizes bacterial membranes in a pH- and oligomerization-dependent manner (Hecht et al. 2004). Being both cationic and very hydrophobic, SP-B utilizes both types of mechanisms. It forms oligomers similar to amoebapore; while lyses negatively charged microorganisms at neutral pH, similar to NK-lysin and granulysin (Poulain et al. 1992, Ryan et al. 2005).

SP-B and SP-C are best known for their capabilities in promoting the spreading and stability of surface films. These hydrophobic proteins are of pathophysiological importance, especially in the case of respiratory distress syndrome (RDS) which is characterized by difficulty in breathing due to the high surface tension of the alveolar. Depletion of SP-B in newborn rabbits was found to cause plasma proteins leaking into the air spaces resulting in high surface tension of the alveolar. SP-C acts as an anchor or linker between the lipid monolayer and soluble interphase, thus lowers the surface tension and increases the adsorption potency of the surfactant.

While SP-A and SP-D are believed to be of minor importance in the surface-tension-reduction properties under physiological conditions, they were implicated in surfactant homeostasis by regulating the production and/or degradation of lipid protein mixture (Korfhagen et al. 1998).

2.1.3 Distribution of SP in ocular surface

As demonstrated by Brauer and his colleagues' work (Brauer et al. 2007), SP-A and SP-D are present within human tear fluid, tissues of the human lacrimal apparatus, corneal and conjunctival epithelial cells. SP-B and SP-C were found to present in the ocular surface and the lacrimal apparatus and secreted into tear fluid. So far functions of SP in ocular epithelium and tear film are mostly speculative and required to be further investigated. From what was known from pulmonary surfactant, these hydrophobic surfactant proteins would presumably be involved in surface activity and maintenance of the tear film associated with the eye blink; SP-C would act as an anchor or linker between the lipid and soluble part of the tear film. As to the hydrophilic proteins, they may work in concert with antimicrobial substances in the innate immune system to fulfill defense functions of the ocular surface.

2.2 SP in ocular barrier functions

2.2.1 Surface tension reduction of tears

The most crucial components for increasing lung surfactant adsorption are SP-A, SP-B and SP-C. Surfactant proteins are also highly important in refining the composition of the surface film during respiratory cycling to maximize dynamic surface tension lowering and re-spreading. SP-A, SP-B and SP-C all have extensive molecular biophysical interactions with phospholipids that contribute to surface activity. SP-A functions as a large octadecamer containing six trimeric subunits, it increases the molecular order and aggregation of phospholipids. It also co-ordinates with SP-B to form tubular myelin, a lipid transport structure (Palaniyer et al. 2001) consists of square tubes of lipid lined with protein (Figure 2). SP-B consists of alpha-helices with disordered links. It can fluidize a monolayer by preventing lipid packing (Krol et al. 2000) which leads to smaller domains in a liquid-condensed state, and makes widespread collapse more difficult. It is the most hydrophobic of all the surfactant proteins. Of all the surfactant proteins, SP-B is the most active in increasing the adsorption and overall dynamic surface activity of phospholipids. Its amphipathic structure allows strong interactions with both the head-groups and chains of phospholipid molecules in films and bilayers, making it a particularly important functional component of endogeneous and exogeneous surfactants. SP-C's main structure feature is an alpha-helix, which is capable of spanning a lipid bilayer. Roles of SP-C include enhancing the rate of lipid adsorption, and promoting multi-layered, stacked structures. It has been suggested that SP-C can act as a lever to move lipids. In SP-C null mice, bubbles of their surfactant are unstable when compressed in the captive bubble surfactometer. Thus,

SP-C may have a stabilizing effect on highly compressed surfactant (Glasser et al 2003).

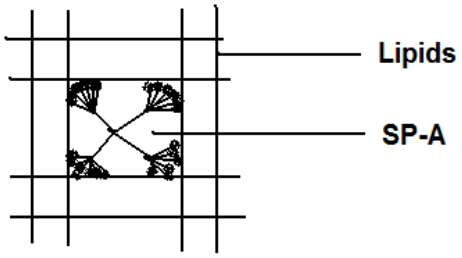


Fig. 2 Tubular Myelin structure

Natural tear film has a three-layered structure (Figure 3). It is composed of an outer oily layer to reduce evaporation of tears. The middle watery layer makes up what we ordinarily think of tears. This layer cleanses the eye and washes away particles that irritate the eye. The inner layer is composed of mucus. The cornea surface in the absence of tear film is hydrophobic, with a low value of the critical surface tension (28 dynes/cm). A solid surface of such a low free energy cannot be completely wetted by an aqueous solution such as tears unless it contains the effective surfactant. Epithelial mucin adsorbs onto epithelium and is capable of maintaining a low interfacial tension at the boundary of the corneal epithelium and the aqueous layer of the tear film through mucin-water interaction (e.g. hydrogen bond formation).

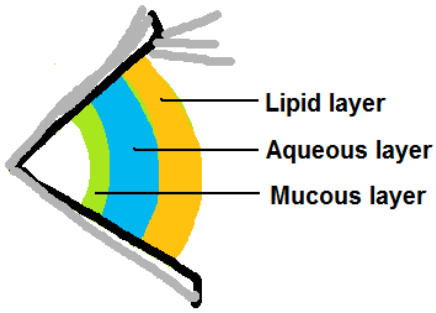


Fig. 3 Tear film structure

Although mucus was once believed to responsible for the physical properties in tears because of its known effect on viscosity and surface tension in mucus solutions, it was discovered that there is little of no mucus in tears, or too little to have the observed effect (Tiffany et al. 1996). It appears that small molecules such as lipids might be responsible. The vast majority of tear-associated lipid is from the Meibomian glands of the eyelid margin, and is brought into contact with the aqueous fluid in formation of the tear film. Some macromolecular components of tears (e.g. lipocalin protein) are known to have lipid-binding capacity. Lipids that bound to lipocalin influence surface tension. Recent discovery of surfactant proteins in the ocular surface suggests that surfactant proteins may also be the major players in reducing surface tension of tear fluid. In artificial tears, sufficient amount of nonionic surfactants is used to provide the solutions with a surface tension of about 38 to 45 dynes/cm (Tiffany et al. 1989), which corresponding to the surface tension of human tears.

2.2.2 Ocular innate immunity

2.2.2.1 Innate and adaptive immune systems

Components of the immune system	
Innate immune system	**Adaptive immune system**
Response is non-specific	Pathogen and antigen specific response
Exposure leads to immediate maximal response	Lag time between exposure and maximal response
Cell-mediated and humoral components	Cell-mediated and humoral components
No immunological memory	Exposure leads to immunological memory
Found in nearly all forms of life	Found only in jawed vertebrates

Table1. Comparison of innate and adaptive immunities

The immune system is composed of two major subdivisions, the innate or non-specific immune system and the adaptive or specific immune system (Table 1). Although both immune systems function to protect against invading microorganisms, they behave differently. Unlike adaptive immune system that requires some time to respond and only reacts with the microbial pathogen that induced the response, innate system is activated upon infection and the reaction is non-specific, which means it responds equally well to a variety of microorganisms. Thanks to innate immunity, animals completely devoid of an adaptive immune system still retain the capacity to resist many infectious agents. Innate immune system is composed of a set of leukocytes and plasma proteins that are able to detect and destroy a broad spectrum of pathogens. The leukocytes include monocytes/ macrophages, dendritic cells, granulocytes (neutrophils, basophils, eosinophils), and lymphocytes (natural killer cells).

Despite their distinct functions, there is interplay between the innate and adaptive immune systems.

2.2.2.2 SP in innate immunity

SP-A and SP-D are known to be able to recognize a broad spectrum of pathogens; therefore they are regarded as “defense molecules” involved in innate immunity. These two collectins have been shown to interact with a number of viruses, bacteria (gram-positive or gram-negative) and fungi. They eliminate microorganisms via several independent approaches as listed below (Figure 4).

1) Direct kill by increasing the membrane permeability thus destabilizing the membrane of gram-negative bacteria;

2) Induce microbial aggregation to neutralize the pathogen;

3) Stimulate immune cell mediated recognition and clearance. SP-A and SP-D have been shown to bind to macrophages, which carry receptors for SP-A and SP-D on their surfaces (Geertsma et al. 1994). This interaction is independent of their prior binding to pathogens. SPs stimulate macrophages in producing reactive oxygen and nitrogen species to enhance the efficiency of uptake and digestion of the malicious microorganisms;

4) Mediate cytokine and chemokine release from the host cells to recruit phagocytes (e.g. neutrophils and macrophages) specifically adapted for defense against microorganisms. To internalize an external particulate, phagocytotic cells first extend portions of their plasma membrane to surround the mass to be engulfed; these cytoplasmic projections then meet and fuse to form an endocytic vesicle containing

the foreign matters. The vesicles then merge with the lysosomes which are sac-like bodies containing digestive and catabolic enzymes.

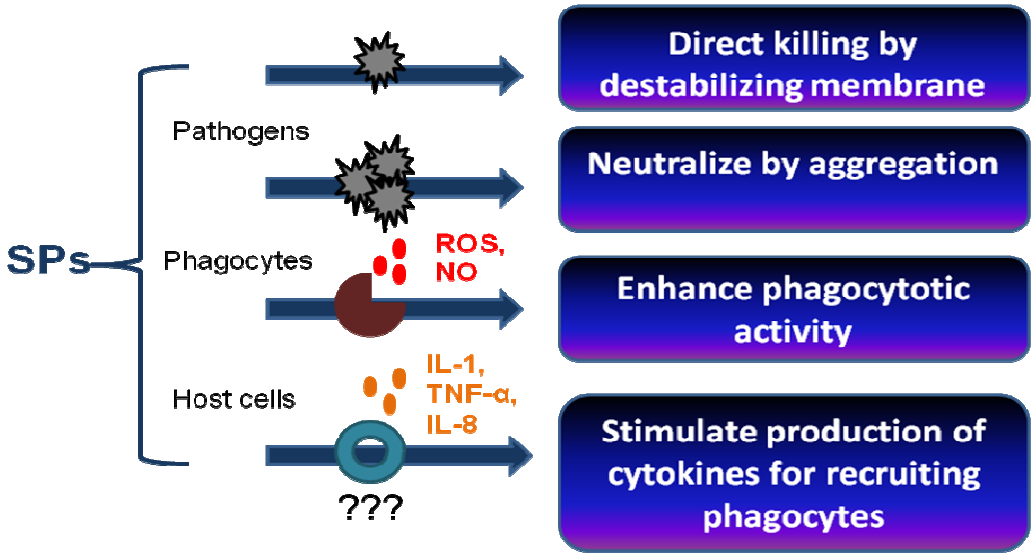


Fig.4 Microbial elimination strategies of SP-A and SP-D

5) Mediate the binding of microorganism to host defense cells. However, opinions are divided concerning how SPs influence the interaction of foreign objects (e.g. apoptotic cell debris, microorganism) with the host cells. Effects of the mediation remain controversial.

Some studies supported the idea that SP-A and SP-D act to obstruct the binding of microorganisms with the surface receptors of the host cells thus inhibit inflammation reactions induced by microbial and their particulates. Lipopolysaccharide (LPS) and peptidoglycan (PGN) are bacterial outer membrane components and potent stimulators of inflammatory responses of host cells. Smooth LPS is composed of lipid A, complete core oligosaccharides and O-antigen; whereas rough LPS

is composed of lipid A and shorter core oligosaccharides. It was shown that both SP-A and SP-D bind LPS receptor CD14, but the mechanisms and the effect of their interactions are different. SP-A binds directly to the protein backbone of CD14 and reduces smooth LPS-induced proinflammtory cytokine (e.g. TNFα) expression. Studies also suggest an interaction of SP-A with rough LPS, but the effect of this interaction remains controversial (Sano et al. 1999). On the other hand, SP-D binds to N-linked oligosaccharides on CD14 and this interaction inhibits both smooth and rough LPS induced proinflammatory cytokine production (Sano et al. 2000). Both SP-A and SP-D were shown to inhibit Toll-like receptor 2 mediated proinflammatory cytokine (e.g. TNFα) release in response to peptidoglycan stimulation.

The above mechanism is contradicted by a recently proposed model that supports the idea that SP-A and SP-D may possess dual biological activities depending on if their CRDs are occupied with infectious particles (Gardai et al. 2003). When their CRDs are in the unbound state, SP-A and SP-D inhibit macrophage activation by binding to signal-regulating protein α (SIRPα). If their CRDs are bound with a ligand of microorganism, SP-A and SP-D would bind to the macrophage-activating receptor calreticulin/ CD91, which subsequently stimulates p38 mediated NF_KB to induce proinflammatory cytokines (Figure 5).

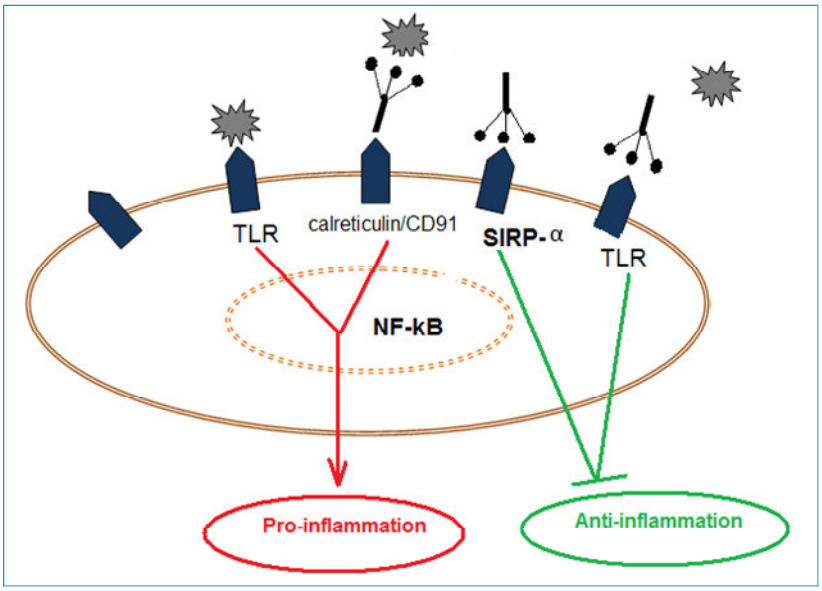


Fig.5 Paradoxical view of SP in mediating inflammation

In spite of the mixed opinions on the immune pathways regulated by SP-A and SP-D, animal models clearly demonstrated that deficiency of either protein results in increased susceptibility to infections (Postle et al. 1999, Cheng et al. 2000, Greene et al. 1999). For example, SP-A and SP-D deficiency has been shown to associate with severe and repeated pulmonary infections in infants; similar findings have been reported in immune-compromised hosts. Restoring the proteins or administration of recombinant proteins is generally believed to reverse defects in microbial clearance and inflammation. Supplementing exogenous SP-A and SP-D therefore would be beneficial to patients with immune defects or surfactant deficiencies.

2.2.2.3 Corneal epithelium and innate immunity

The cornea is the clear, protective outer layer of the eye, pretty much like a crystal clear window that lets us to look through. Transparency is achieved through a lack of blood vessels, pigmentation, and keratin, and through a tightly layered organization of the collagen fibers. Along with the sclera (white of the eye), cornea serves as a barrier against invading pathogens that can harm the eye. It is also capable of filtering out some amounts of the sun's ultraviolet light. Tiny blood vessels around the outer edge of the cornea (limbus), along with the tear fluid, provide the cornea with necessary nourishment. The cornea is about 1.5 mm thick and composed of 5 layers: epithelium, Bowman's membrane, stroma, Descement's membrane and the endothelium (Figure 6).

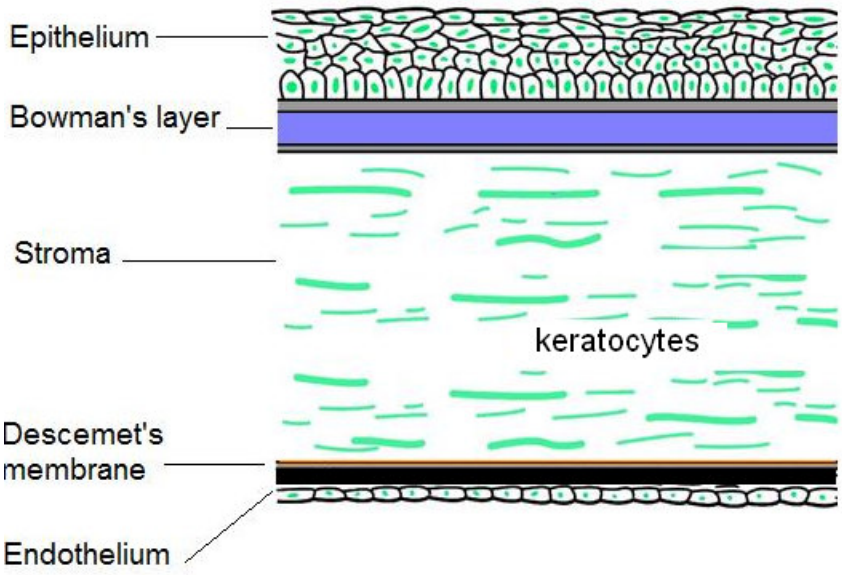


Fig.6 Structure of corneal epithelium

Corneal epithelium is composed of 5-7 layers of non-keratinized cells and has a transparent appearance. These cells produce hydrophilic

molecules called mucins at the surface to hold the tear film in place, which keeps the eye moisturized and protected from invading pathogens. The epithelium also prevents water from entering the cornea which can disrupt collagen fibers in the stroma. Boman's membrane lies beneath the epithelium and provides an additional layer of protection. The stroma is the thickest layer (about 0.5mm) and composed of collagen fibers arranged in a strictly paralleled fashion. This special organization gives the eye clarity, allowing 99% of light to pass through without scattering, as well as strength and resilience. Excessive water absorption by this layer (called cornea edema) can disrupt collagen fiber organization, cause a hazy and opaque appearance, and reduced vision. Descemet's membrane lies between the stroma and the endothelium. Endothelium is a single cell layer separates the cornea from the aqueous humor- the clear fluid in the front chamber of the eye; it pumps water from the cornea, keeping it clear.

Epithelium is more than a physical barrier; it is an active participant in the innate host response to infection (Savkovic et al. 1997, Wick et al. 1991, Monick et al. 2003, Johnston et al. 1998, Meusel et al. 2003, Nadeau et al. 2002, Bals et al. 2004). In response to pathogen challenge, epithelial cells can produce and secret various antimicrobial factors (e.g. β-defensin antimicrobial peptides) and proinflammatory cytokines (IL-1, TNFα, IL-6 and IL-8) which orchestrate neutrophil and monocyte infiltration and subsequent activation of cell-mediated immune defenses.

Production and secretion of some of these innate defense factors depends upon epithelial recognition of common microbial surface antigens often referred to as pathogen-associated molecular pattern (PAMPs). Examples of PAMPs include lipopolysaccharide (LPS) (cell membrane

component of Gram-negative bacteria), peptidoglycan (PGN) (cell wall component of Gran-positive bacteria) and flagellin; they are recognized by cellular receptors such as the revolutionary conserved Toll-like receptors (TLRs). Binding of microbial antigens to TLRs results in the activation of Mitogen-activated protein kinases (MAPK) related intracellular signaling pathways that leads to proinflammatory cytokines and chemokines expression and secretion. Derived from neutral crest tissue, corneal stroma keratocytes and epithelium are able to produce soluble and diffusible chemokines, such as IL-8 as the direct mediator of leukocyte infiltration. Neutrophils migrate along the gradient of chemokines to the inflamed sites.

In vascularized tissue such as skin, inflammatory mediators and leukocytes arrive at the inflamed sites directly from blood vessels. However the central cornea has no blood vessels and is also absent of Langerhans cells. Limbal epithelium, the transitional zone of the cornea and conjunctiva, contains melanocytes, Langerhan cells and an underlying network of blood vessels in the limbal stroma. Eyelid also contains a rich supply of blood vessels and was recently believed to contribute to neutrophil recruiting (Sloop et al. 1999). Soluble inflammation mediators secreted from epithelial cells diffuse through the tear fluid and elicit an immediate response in the limbus, bulbar conjunctiva or even the eyelid; consequently the blood supplies in these regions bring neutrophils to the local sites where inflammation occurs, and tear fluid brings neutrophils to the corneal surface.

2.2.2.4 Tissue injury and repair

Corneal epithelial injury

Surgical procedures (e.g. photorefractive keratectomy PRK, or laser in situ keratomeliusis LASIK), microbial infection or simple mechanical compression (e.g. by contact lens) induces keratinocyte apoptosis and necrosis in epithelium or stroma. The cornea absorbs more UV-B radiation than any other ocular tissue, thus acts as a filter and protects the lens and retina from UV-induced damage (Kolozsvari et al. 2002). UV irradiation from sun exposure is sufficient to induce corneal epithelial cell death. Photokeratitis (snow blindness or welder's flash) is a painful but transient inflammatory condition induced by acute UV radiation. Evaluation of UN-irradiated pre-corneal tear film may reveal a discharge of debris that appears to be particles of disintegrated surface cells, and whole cells shed from the surface. Epithelial shedding was shown to be accelerated by UV exposure (Ren et al. 1994).Injured ocular epithelium is more susceptible to pathogen invasion and infections.

Wound healing

Superficial wounds involve a loss of part of the epithelium; deep wounds extend into or through the stroma. Apoptotic cells or their debris released into the tear fluid serve as danger signals that trigger an immediate secretion of proinflammatory cytokines as interleukin IL-1 and tumor necrosis factor TNFα from the cells. These cytokines act as master modulators of many of wound healing events by exerting effects on the deeper layers or other ocular regions (e.g. limbus, eye lid) through diffusion into injured epithelium barrier or tear fluid.

IL-1 is the primary regulator of growth factors [e.g. hepatocyte growth factor (HGF), keratinocyte growth factor (KGF) and platelet derived growth factor (PDGF)] that responsible for cell proliferation, migration and differentiation. Cells that have left the limbal basal layer become differentiated and express keratin 12, specific corneal epithelial differentiation maker (Liu et al. 1994). Injured corneal epithelium is gradually replenished with cells migrating from the periphery limbal epithelium and the basal layers. This cell turnover continues in an orderly fashion until the denuded areas are fully resurfaced with the migrated cells. Furthermore, IL-1mediates wound remodeling by upregulating metalloproteases (MMPs) and other enzymes that control the synthesis, breakdown and cross-linking of collagen. The remodeling process which normally takes months to years eventually restores the cornea to normal transparency at the site of injury.

Induced by IL-1 and TNFα, keratinocytes and corneal epithelial cells produce IL-8 chemokine and monocyte chemotactic protein (MCP-1) to attract T-cells and non-specific macrophages/ monocytes to the

inflamed sites via the limbal and eye lid blood supplies, as well as from the tear fluid. Macrophages act almost exclusively by phagocytosis, whereas neutrophil granulocytes are more effective in pathogen elimination by secreting toxic mediator myeloperoxidase that is able to kill the pathogens. IL-8 has also been shown to stimulate α-smooth muscle actin production in human fibroblasts and cause the wound to contract and close more rapidly (Feugate et al. 2002). Furthermore, IL-8 is chemotactic for fibroblasts and accelerates their migration as well as deposition of matrix components such as fibronectin and collagen I during wound healing (Kuhlmann et al. 2009). Fibronectin from the tear film can also deposit on the denuded corneal surface and may facilitate cell adhesion and re-epithelialization during healing.

The general process of corneal epithelial wound healing is summarized in Figure 7.

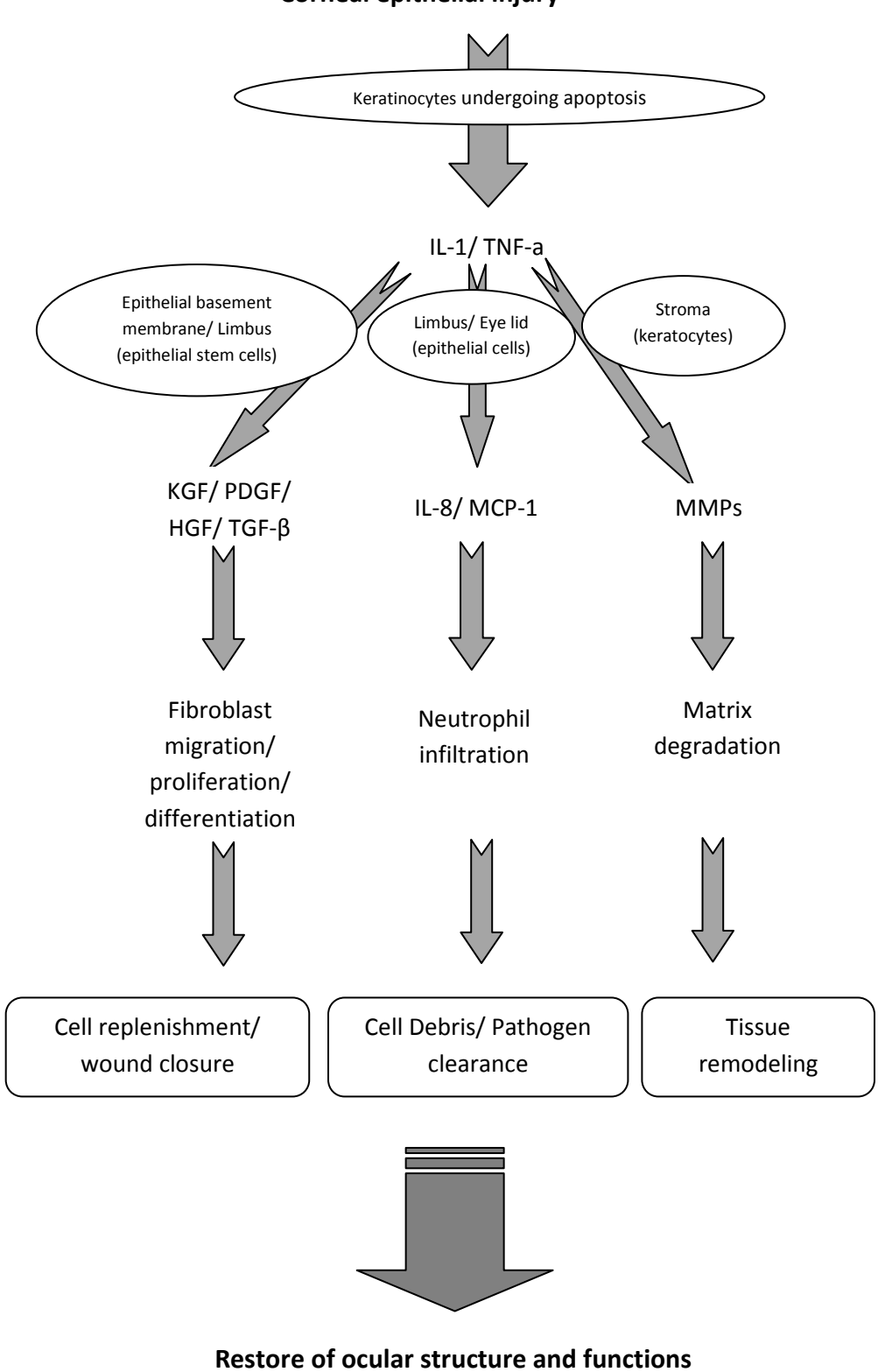

Corneal epithelial injury
Keratinocytes undergoing apoptosis
IL-1/ TNF-a
Epithelial basement membrane/ Limbus (epithelial stem cells)
Limbus/ Eye lid (epithelial cells)
Stroma (keratocytes)
KGF/ PDGF/ HGF/ TGF-β
IL-8/ MCP-1
MMPs
Fibroblast migration/ proliferation/ differentiation
Neutrophil infiltration
Matrix degradation
Cell replenishment/ wound closure
Cell Debris/ Pathogen clearance
Tissue remodeling
Restore of ocular structure and functions

Fig. 7 General process of corneal epithelial wound healing

3 SP in modulating corneal epithelial innate immunity

3.1 Objectives

Surfactant proteins (SP) have long been acknowledged for their important roles in maintaining lung functions. Although we have gained a wealth of knowledge about SPs through decades' research in pulmonary tissue, some fundamental questions still remain unanswered. Currently, opinions are divided concerning their influence on inflammatory cytokine production in response to an injury or pathogen attack. Moreover, hydrophobic proteins SP-B and SP-C are traditionally thought to play a minor role in host defense and immunity therefore their studies in the area have been very scarce. On top of that, even much less is known about how SP behave in tissues other than lung such as the ocular tissue, where their presence was discovered just recently.

To shed light on the involvement SP in inflammation reactions in ocular epithelium after exposure to harmful stimulus, the following aims were set to this thesis:

1. To evaluate the effect of SP depletion on inflammatory cytokine production by human corneal epithelial cells in response to UV irradiation and PGN challenge.

2. To evaluate the effect of exogenous recombinant SP on UV and PGN-induced cytokine production.

3. If findings from the above studies are supportive, proceed to determine the capability of exogenous SP in reversing the change in immunological response observed in SP-depleted cells.

3.2 Materials and methods

3.2.1 Cell culture

HCE-2, an adenovirus SV40 immortalized corneal epithelial cell line (CRL-11135, American Type Culture Collection, MA, VA) was cultured according to manufacturer's recommendations. Upon thawing, cells were maintained in keratinocyte serum free medium (KSFM, Gibco) containing 0.05 mg/ml bovine pituitary extract (BPE), 5 ng/ml human recombinant epidermal growth factor (EGF), 500 ng/ml hydrocortisone and 0.005 mg/ml bovine insulin at 37^{o}C in a 5% CO_2 humidified atmosphere. Medium was renewed twice per week until cells are ~80% confluent. To facilitate cell attachment, flasks were pre-coated with ECM gel (E1270 from Sigma).

NHEK, normal human neonatal epidermal keratinocytes (Invitrogen) was cultured according to manufactory's recommendations. Upon thawing, cells were cultured in fully supplemented growth medium (KBM supplemented with HKGS, Gibco) containing 0.2 % v/v of BPE, 0.2 ng/ml of EGF, 5 µg/ml of transferring, 5 µg/ml of insulin, 0.18 µg/ml hydrocortisone. Medium was renewed every other day until cells are ~ 70% confluent.

For passaging both HCE-2 and NHEK, sub-confluent cells were detached from the flask by 0.05% w/v Trypsin/ 0.53mM EDTA, centrifuged at 8000 g for 10 minutes, re-suspended and subsequently seeded in a multi-well cell culture plate at a density of 20,000 cells/ cm^2. Cells were allowed to settle for 24 hours before the treatment. To support the function of surfactant proteins, culture medium that used for treatment was supplemented with 0.5 mM calcium chloride.

3.2.2 Short interfering RNA (siRNA)-mediated gene silencing

Commercial siRNA targeting SP-A, SP-B, SP-C or SP-D (Santa Cruz Biotech, Santa Cruz, CA) is a pool of three target specific 19-25 nucleotide-long double stranded RNA molecules with 2-nt 3' overhangs on each end. After entering the cells, siRNA causes cleavage of target mRNA and therefore leads to inhibition of gene expression. siRNA was transfected to cells according to manufactory's recommendations. Briefly, healthy and sub-confluent cells were cultured in serum and anti-biotic free normal growth medium containing a mixture of siRNA duplex and a lipid-based transfection reagent (Santa Cruz) for 5-7 hours, followed by additional 24 hours culture in further diluted transfection mixture. Control cells were handled in the same manner except that they were transfected with siRNA consists of a scrambled sequence that will not lead to the specific degradation of any known cellular mRNA. Cells were subjected to specific treatment within 24 to 72 hours after transfection. Effectiveness of the transfection was visually demonstrated by FITC-conjugated siRNA control (Figure 8). SP expression and production in knockdown cells were reduced by 30% evidenced by ELISA and PCR results.

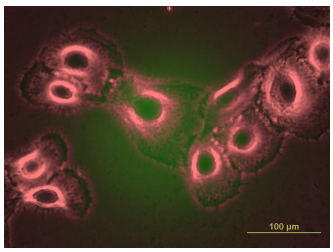


Fig.8 Microscopic image of HCE-2 transfected with FITC-conjugated siRNA control

3.2.3 UV exposure of cells

A UV box equipped with two UV-B fluorescent tubes (UBL FSX 24T12/UV-B-HO, National Biological Corp., Beachwood, OH) was used as light source for irradiation. Irradiance was measured by a radiometer (IL-1700, UV Process Supply, Chicago, IL) that equipped with the necessary probe components include detectors, filters, and input optics. Prior to UV irradiation, sub-confluent cells were switched into Hanks Buffered Saline Solution (HBSS). Cell culture plate cover was replaced with Reynolds 914 film (alcohol sterilized) to allow better penetration of UV rays. Cells were irradiated at an intensity of 0.24 mW/cm^2 for 125 seconds to achieve a dose of 30 mJ/cm^2. After exposure, HBSS was aspirated and cells were cultured in normal medium with or without the presence of effectors (e.g. recombinant surfactant proteins). After cultured for additional 24 hours, supernatant was harvested and cells were rinsed with ice-cold Phosphate Buffered Solution (PBS). Supernatant and cells were assayed immediately or stored at -70^oC until further analysis. Unexposed control plate was handled identically to the others except that it received no UV irradiation.

3.2.4 Cytokine induction and quantification

HCE-2 or NHEK cells were induced for IL-8 production by UV irradiation or PGN (Cat # 77140, Sigma Aldrich, St. Louis, MO). To induce IL-8 by UV, cells were exposed to 30 mJ/cm^2 of UV-B as previously described. Cells were then cultured in medium with or without rhSP. For PGN induction, 20 µg/ml of PGN suspension was made in culture medium, bath-sonicated for 20 minutes and added to the cells. Cells were cultured in PGN suspension with or without rhSP for 24 hours. Supernatant was subsequently collected and analyzed for IL-8 and/ or TNF-α secretion with commercial ELISA kits (R&D systems, Minneapolis, MN) according to manufacturer's instructions. Cells were rinsed with ice-cold Phosphate Buffered Solution (PBS) and were immediately lyzed for quantification assays (e.g. protein or RNA quantification) or stored at -70^{o}C until further analysis.

3.2.5 RNA isolation and Taqman® Real-Time PCR

Total RNA from the cells was extracted using Qiagen RNeasy Mini kit according to manufacturer's instructions. Concentration and purity of the RNA preparations were assessed by measuring the absorbance at 260 nm and 280 nm by a spectrophotometer (NanoDrop ND 8000; Bioscience, San Luis Obispo, CA). Reverse Transcription of 1-2 µg total RNA was performed at 37^{o}C for 2 hours and 85^{o}C for 5 minutes using ABI High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster city, CA). 10 µl of 1st strand cDNA product was brought to a volume of 25 µl containing Taqman Universal PCR Master Mix, Taqman primer and probe sets for control gene GUSB (beta glucuronidase) and target gene IL-8 (ABI Taqman assay # Hs99999034_m1). Quantitative PCR was performed by using ABI StepOne plus real time PCR equipment under the following conditions: 50 cycles at 95^{o}C for 15 seconds and 60^{o}C for 1 minute. The oligonucleotide sequences and the specific fluorescence labeled DNA probes were selected to span exon junctions of the target genes. All PCR reactions were performed in triplicate. The comparative C_t method was used to quantify transcripts, and the expression level was normalized to that of the human housekeeping gene GUSB or GAPDH. Normalizing the results to the GUSB expression provided results identical to those obtained using GAPDH (data not shown).

3.2.6 Intracellular surfactant protein quantifications

Production of surfactant proteins by cells was determined by commercial ELISA kits specifically designed for each type of SPs (Cusabio Biotech, Wuhan, China) with detection sensitivities of 3.12 ng/ml, 1.95 ng/ml, 2.5 ng/ml and 1.56 ng/ml for SP-A, SP-B, SP-C and SP-D respectively. While secreted proteins were below the detection limit, intracellular SP-B, SP-C and SP-D were readily detected in cell lysates containing at least 0.2 million cells per ml. There were some technical difficulties in measuring SP-A protein or mRNA. Cell lysates were obtained by collecting the first flow-through during total RNA extraction (RNeasy Mini kit, Qiagen Inc., Valencia, CA). Total protein concentration in cell lysates were determined (BCA, Pierce) and was used to normalize SP contents. Purified, non-tagged recombinant surfactant proteins provided by the kits contain the sequences of full-length matured proteins and were added to the conditioned medium. SDS-PAGE analysis confirmed the sizes of rhSP-A, rhSP-B, rhSP-C and rhSP-D as 25KD, 9KD, 4KD and 40KD respectively, which are quite consistent with that of the native proteins.

3.2.7 Expression results and statistics

At least three wells were used for each group of samples in all experiments, which were repeated twice. Data for cytokine secretion/ expression are expressed as percentage of untreated control ± standard error. Untreated controls are cells cultured in normal medium without inducers or testing compounds of any kind. All other data are expressed as means ± standard error. The various assay conditions were compared to the untreated/ non-induced condition using Student's *t* test, probability values less than 0.05 were considered to be statistically significant and marked with * in graphs.

3.3 Results

3.3.1 Effect of SP depletion on cytokine production by HCE-2 cells

For verification purposes, SP protein production in cells transfected with SP siRNA was analyzed (Figure 9). Control cells were transfected with control siRNA. After transfection, cells were cultured in regular growth medium for additional 48 hours. SP contents in cell lysates were determined by ELISA. Total proteins in cell lysates were determined by BCA to normalized SP concentrations.

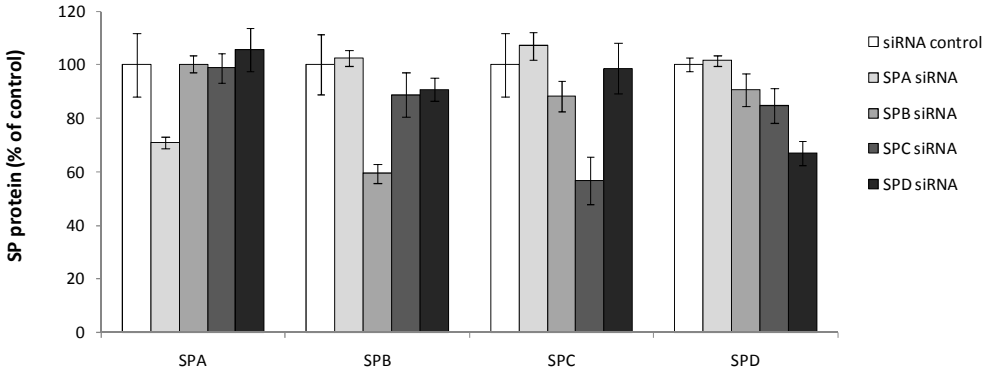


Fig. 9 SP protein in SP-silenced HCE-2 cells

The following experiment was designed to determine if UV/ PGN induction of IL-8 is affected by the inhibition of SP expression. Cells were transfected with siRNAs targeting SP-A, SP-B, SP-C or SP-D, then exposed to either 30 mJ/cm^2 of UV-B or 20 μg/ml of PGN. After 24 hours, supernatant and cells were collected for IL-8 secretion and transcription quantification. Data are the mean ± SE of results in three independent experiments and are expressed as % of untreated, non-induced cells transfected with control siRNA. Production of UV-or PGN-induced IL-8

was compared between cells transfected with SP-siRNA and those transfected with control-siRNA. * Probability < 0.05 based on Student's two-tailed *t*-test.

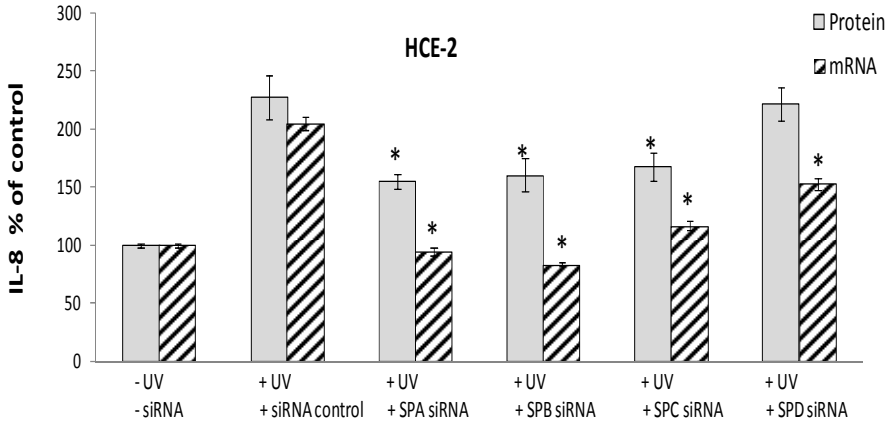


Fig. 10(a) Effect of SP silencing on UV induced IL-8 secretion and transcription

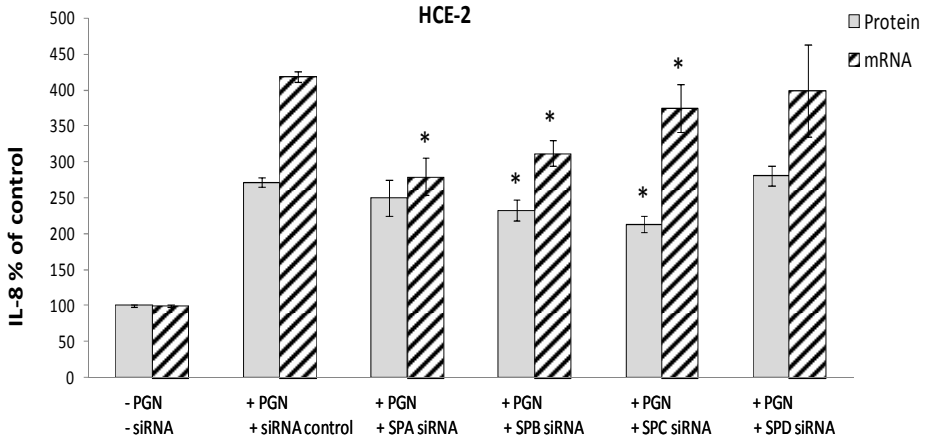


Fig. 10 (b) Effect of SP silencing on PGN-induced IL-8 secretion and transcription

siRNA transfection effectively reduced SP protein by 30-40%. UV and PGN elicited an increase of IL-8 secretion and transcription by at least two folds. The induction was suppressed by knockdown of SP-A, SP-B or SP-C gene expression. Interestingly, inhibition of SP-A and SP-D expression had the strongest and weakest effects on suppressing IL-8 production respectively. Protein and transcription levels of SP were decreased by 30-40% in silenced cells as evaluated by ELISA and PCR analysis. Cytotoxicity analysis revealed 20-30% cell death after acute UV-B irradiation; but exposure to PGN for 24 hours didn't seem to affect cell viability (longer incubation may cause significant cell death). 10-20% cell loss was observed in cells transfected with siRNA targeting SP-A, SP-B and SP-D, but not SP-C.

3.3.2 Effect of recombinant SP on cytokine production by HCE-2 cells

This experiment was to determine if recombinant SPs affect UV and PGN in induction of IL-8 in HCE-2 cells. HCE-2 cells were exposed to either 30 mJ/cm^2 of UV irradiation or 20 µg/ml of PGN; cells were grown in medium containing 50 ng/ml of rhSP-A, rhSP-B, rhSP-C or rhSP-D for 24 hours. Supernatant and cells were subsequently collected for IL-8 protein and mRNA analysis. Data are the mean ± SE of results in three independent experiments and are expressed as % of untreated/ non-induced cells. After exposed to either UV or PGN, production of IL-8 in cells grown in the presence of rhSP was compared to those grown without rhSP. * $p < 0.05$ based on Student's two-tailed *t*-test.

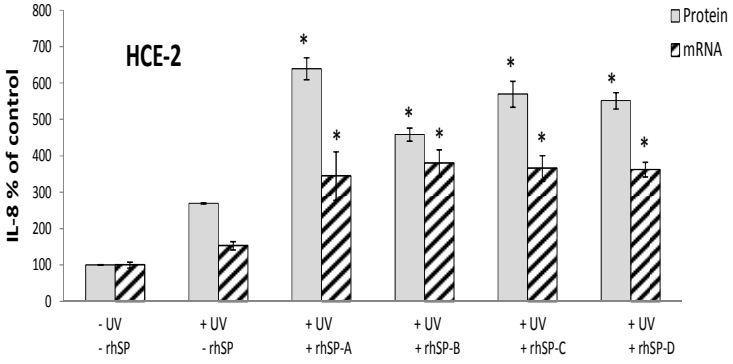


Fig.11 (a) Effect of 50 ng/ml of recombinant SP on UV-induced IL-8 secretion and transcription

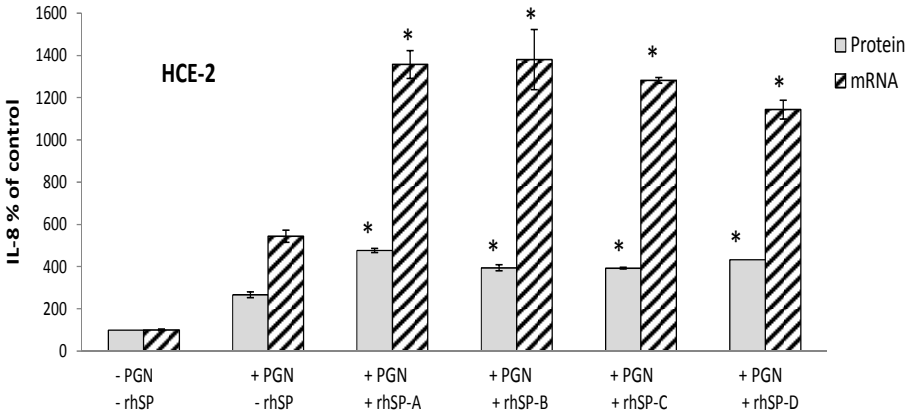


Fig. 11(b) Effect of 50 ng/ml of recombinant SP on PGN-induced IL-8 secretion and transcription

Data showed that rhSP significantly boosted IL-8 protein and mRNA levels in UV/ PGN-induced cells. rhSP showed a remarkable stimulatory effect on IL-8 transcription in PGN-induced cells.

3.3.3 Dose effect of recombinant SP on UV/ PGN induced IL-8 secretion by HCE-2 cells

Cells were exposed to 30 mJ/cm^2 of UV or 20 µg/ml of PGN, and grown in culture medium added with rhSP of various concentrations for 24 hours. Supernatant was determined for IL-8 concentration. (a) Effect of rhSP on UV induced IL-8 secretion; (b) Effect of rhSP on PGN induced IL-8 secretion. Attached cells were lyzed and determined for total protein concentration (BCA, Pierce). Concentration of secreted IL-8 was normalized to total protein in cell lysates. Data are the mean ± SE of results in three independent experiments and are expressed as % of untreated and non-induced cells.

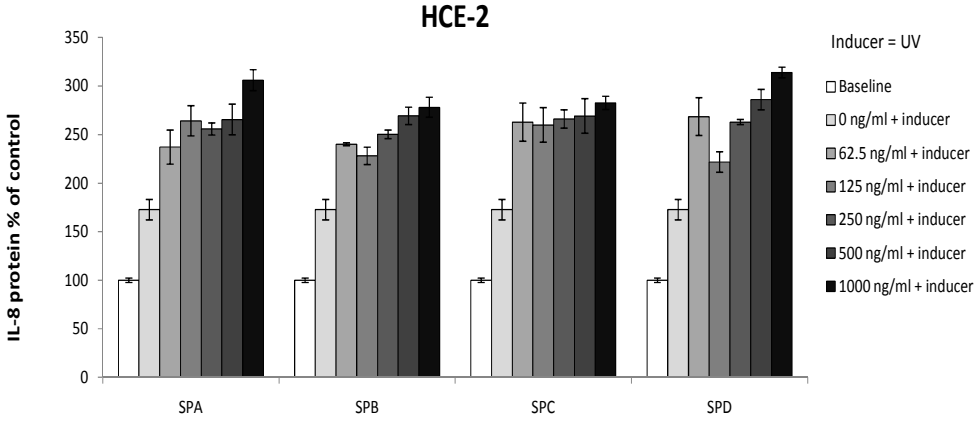


Fig. 12(a) Dose-effect of recombinant SP on UV-induced IL-8 secretion

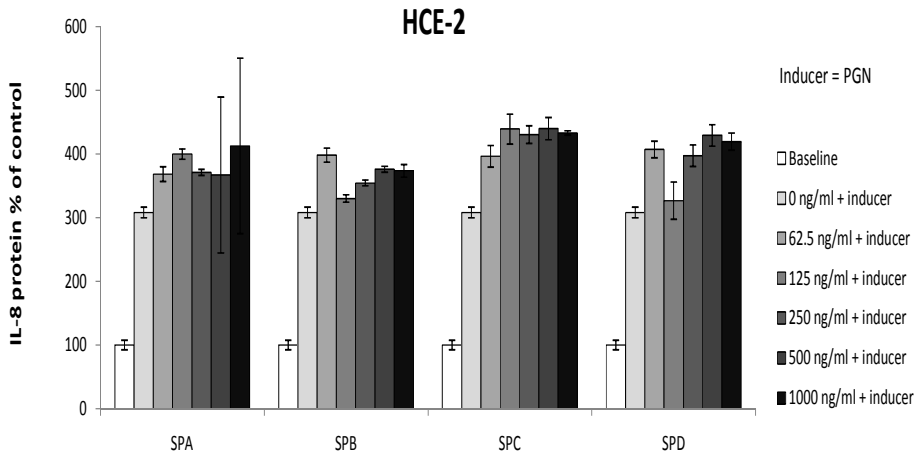


Fig. 12(b) Dose-effect of recombinant SP on PGN-induced IL-8 secretion

The result showed that rhSPs facilitated UV and PGN in inducing IL-8 at all tested concentrations. Dose effect relationship between rhSP and IL-8 was not as strong as expected possibly due to the relative small testing range. It is interesting to note that rhSP-B or rhSP-D of a lower dosage (e.g. 62.5 ng/ml) resulted in a higher than expected induction and this occurred in UV as well as PGN induced cells.

3.3.4 Effect of recombinant SP on induced IL-8 production in SP-silenced HCE-2 cells

The experiment was designed to further confirm previous findings. HCE-2 cells were transfected with a cocktail of siRNAs to simultaneously inhibit the expression of all four types of SP. SP-silenced cells were then exposed to 30 mJ/cm^2 of UV, followed by 24 hours culturing in medium containing 50 ng/ml of rhSP-A, rhSP-B, rhSP-C or rhSP-D. Supernant was analyzed for IL-8 secretion. Cells were lyzed and assayed for total protein concentrations (BCA, Pierce). IL-8 concentraion was normalized to total protein in cell lysates. As expected from previous findings, SP knockdown drastically reduced the IL-8 production in UV-exposed cells, whereas SP supplementation was able to reverse this effect.

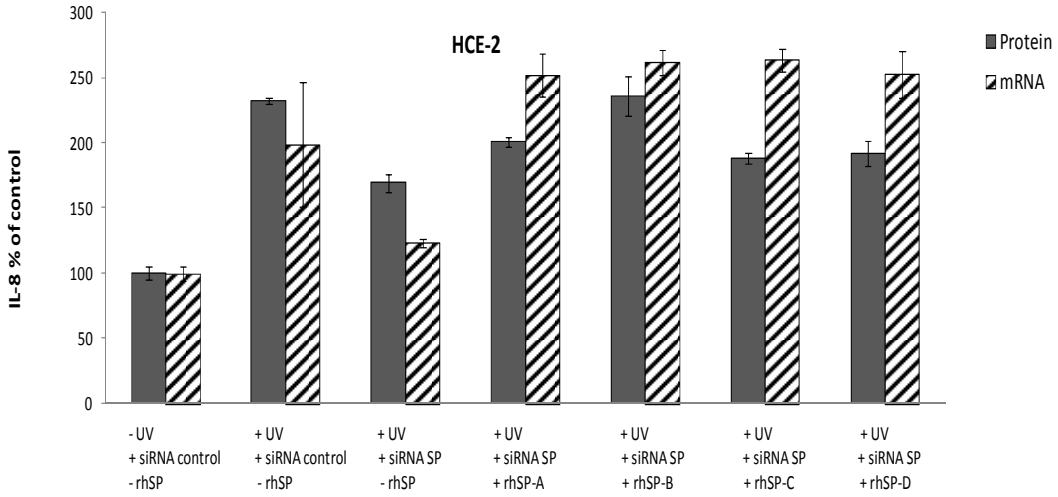


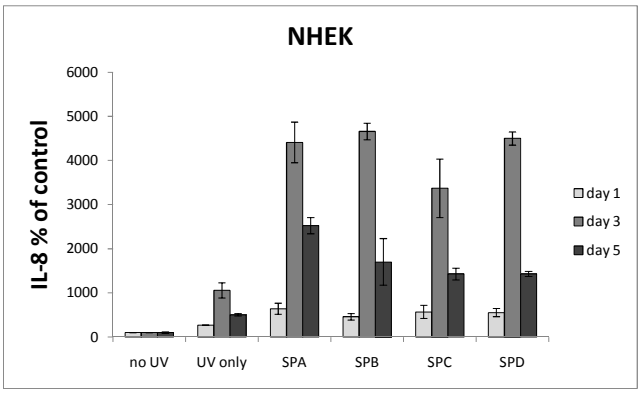
Fig.13 rhSP restored the effect of UV on IL-8 induction in SP-silenced cells. Data are the mean ± SE of results in three independent experiments and are expressed as % of untreated and un-induced cells transfected with control siRNA

Presence of surfactant proteins in skin epidermis was also reported (Mo et al. 2007). So far studies of SP in skin cells have been very scarce. In the present work, a series of experiments have been designed to explore the inflammatory response of epidermal keratinocytes.

3.3.5 Effect of recombinant SP on UV-induced IL-8 and TNF-α secretion in NHEK cells (a 5-day study)

Cytokine secretion was tracked for 5 days after the treatment. Cells were treated in the similar way as HCE-2 cells, except that parallel experiments were set up for studying additional time points. After exposed to 30 mJ/cm^2 of UV, cells were treated with 50 ng/ml of rhSP during the first 24 hours; supernatant and cells were collected at specific time points (e.g. 1, 3 , 5 days after the treatment); culture medium was renewed every 2 days for the remaining plates. Supernant was analyzed for cytokine secretion. Cells were lyzed and assayed for total protein concentrations (BCA, Pierce). Cytokine concentraion was normalized to total protein in cell lysates. Data are the mean ± SE of results in three independent experiments and are expressed as % of untreated and non-induced cells.

Results showed that secreted TNFα in cells cultured in control medium was transiently secreted after UV exposure and peaked by 24 hours while IL-8 secretion was somewhat delayed; peaked by 72 hours. Exogenous SP dramatically enhanced both TNFα and IL-8 secretion.



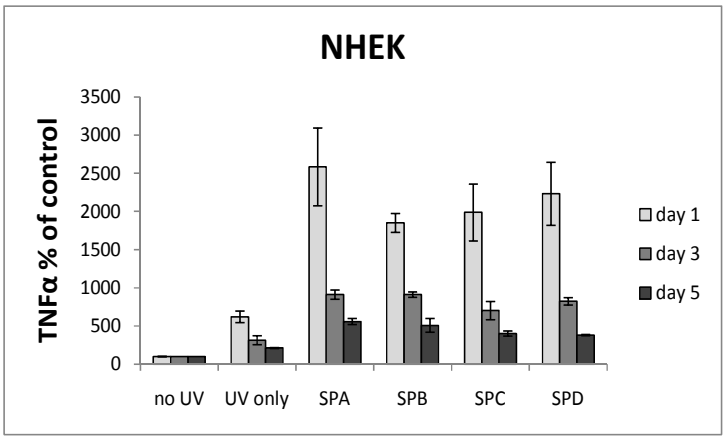


Fig. 14 (a) Effect of 50 ng/ml of recombinant SP on UV induced IL-8 (Top) and TNFα (bottom) secretion in NHEK cells over 5-day culturing

3.3.6 Effect of recombinant SP on PGN-induced IL-8 and TNF-α secretion in NHEK cells (a 5-day study)

Similarly, effect of rhSP on PGN-induced cytokine production was studied. Cells were cultured in medium containing 20 µg/ml of PGN, as well as 50 ng/ml of rhSP for 24 hours. Parallel experiments were set up for studying additional time points. Cells were exposed to 50 ng/ml of rhSP during the first 24 hours; supernatant and cells were collected at specific time points (e.g. 1, 3 , 5 days after the treatment); culture medium was renewed every 2 days for the remaining plates. Supernant was analyzed for cytokine secretion. Cells were lyzed and assayed for total protein concentrations (BCA, Pierce). Cytokine concentraion was normalized to total protein in cell lysates. Data are the mean ± SE of results in three independent experiments and are expressed as % of untreated and non-induced cells.

Results showed that PGN challenged cells had persistantly high secreteion of TNFα and IL-8 over five days in both control and treated cells. Once again, exogenous SP dramatically enhanced both TNFα and IL-8 secretion.

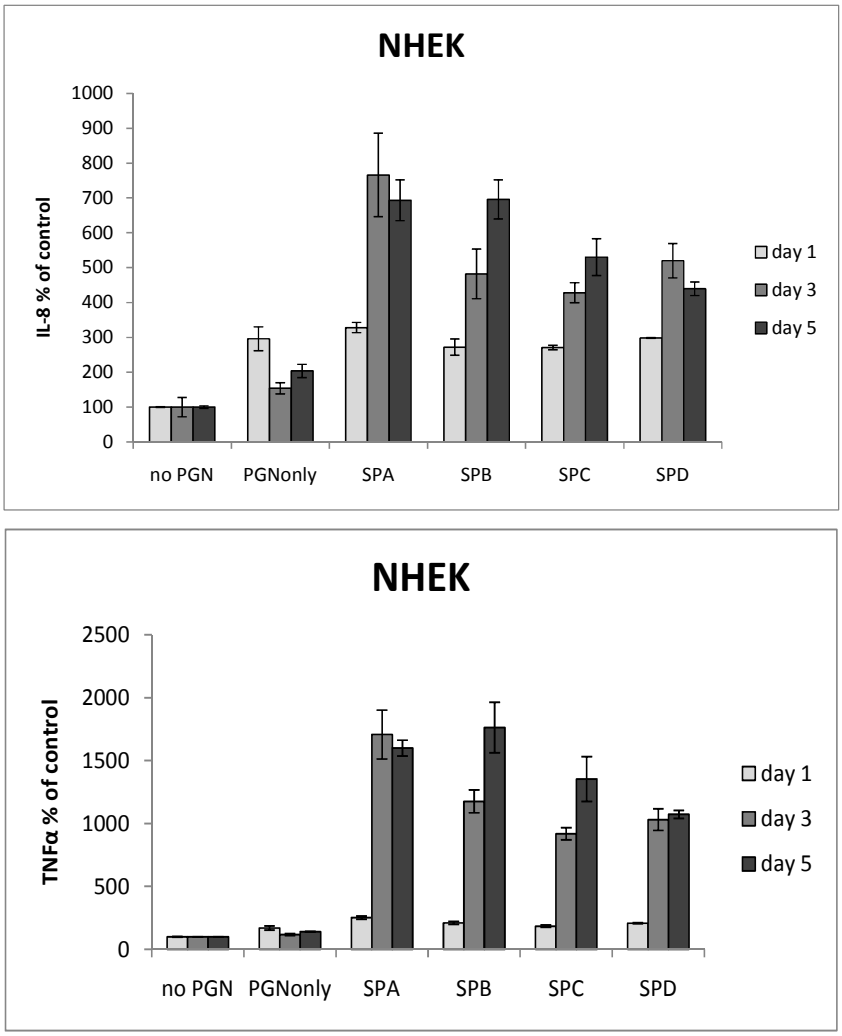


Fig.14 (b) Effect of recombinant SP on PGN- induced IL-8 (Top) and TNFα (bottom) secretion in NHEK cells over 5-day culturing

3.3.7 Effect of external stimulus on intracellular SPs of NHEK (a 5-day study)

This experiment was designed to study the effect of UV irradiation, PGN and LPS on SP production in NHEK cells. Cells were exposed to a type of stimuli and were collected after 24 hours culture. Intracellular SP-B, SP-C and SP-D were determined by ELISA kits (Cusabio, Wuhan, China) specifically designed for each type of the SPs. SP quantity was normalized by total protein concentrations in cell lysates (BCA, Pierce Biotech.). Data are the mean ± SE of results in three independent experiments and are expressed as % of untreated and non-induced cells.

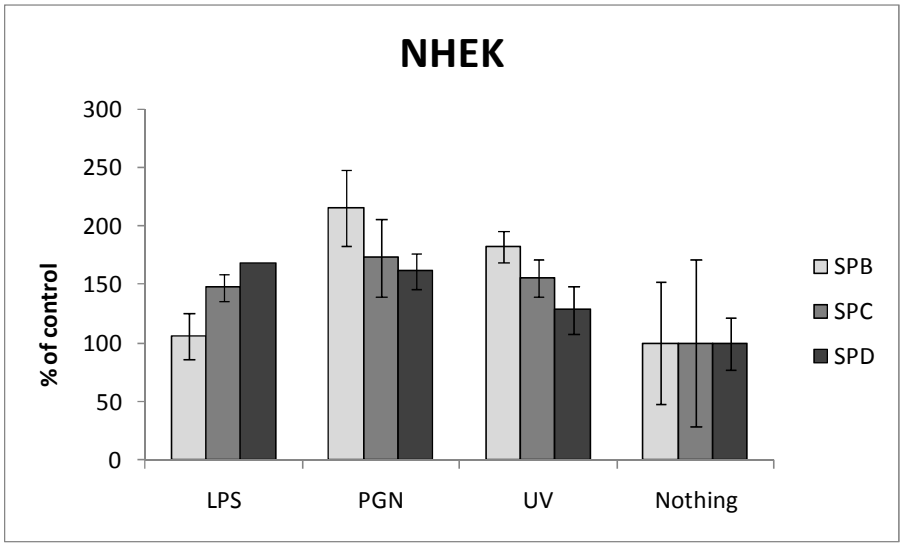


Fig.15 Effect of LPS/ PGN/ UV on intracellular SPs

Intracellular SP-A of control cells was too low to be reliably detected. All tested external stimulus enhanced intracellular levels of SP-B, SP-C and SP-D. Stimulatory effect of LPS appeared to be strongest on SP-D, weakest on SP-B. PGN has the strongest stimulatory effect on SP-B, weakest on SP-D. Effect of UV was somewhat similar to PGN.

3.3.8 Ingestion of conjugated- S. aureus bacteria by HCE-2 cells

Cells were exposed to 1 mg/ml of conjugated S. aureus bacterial particles (PHrodo^{TM} S. aureus BioParticles® conjugate, Molecular Probes, Eugene, OR) for 3 hours. The bacterial particles are conjugated to a fluorogenic dye that dramatically increases in fluorescence upon ingestion as the pH of its surroundings becomes more acidic. After removal of the bacteria, cells were examined by a fluorescence microscope (Olympus model BX2, Center Valley, PA).

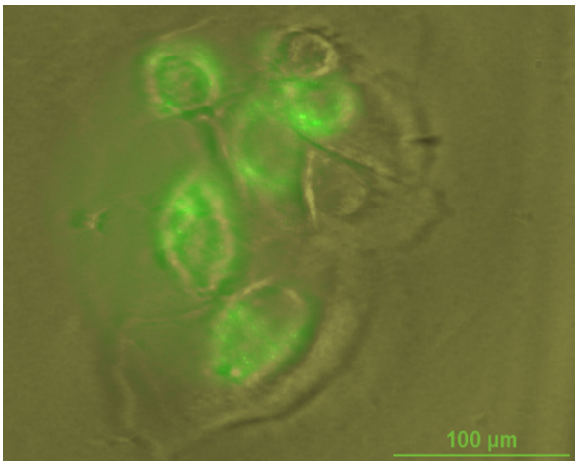


Fig.16 Adhesion and internalization of bacteria by the cells

3.4 Discussions

3.4.1 Methodological aspects

Due to the difficulty in acquiring primary corneal epithelial cells, a commercial immortal corneal epithelial cell line (HCE-2) was used in the present study. HCE-2 has been widely accepted as a model in human corneal epithelial research as it remains morphological, biochemical and functional characteristics of primary corneal epithelia cells (Offord et al. 1999, Araki-Sasaki et al. 1995). In this study, HCE-2 was found to respond reasonably well to a range of stimulations and produced cytokines and growth factors such as IL-8, TNF-α, IL-1α, IL-1β, VEGF, MMP-1 etc (some data not shown). However differences in surface receptors expression in HCE-2 and primary cells has been reported (Benko et al. 2008). Therefore, confirmation of the present findings on primary cells may be pursued in future studies.

Short interfering RNA (siRNA) mediated gene silencing is an effective tool in studying functions of a specific gene or protein. As long as the gene or protein of interest does not profoundly affect the mortality of the host cells and has a relatively short turn-over time, the effect observed in silenced cells should provide good indications of the physiological functions of a particular gene or protein under study. Effect of recombinant proteins can also be evaluated to verify the findings of a gene silencing study.

UV irradiation and PGN were used in the present study to mimic the common environmental stimulus that corneal epithelium exposed to. They are known to induce IL-8 via MAPK signaling pathways (Kumar et al. 2004). UV irradiation causes cell shedding into the tear fluid and PGN is recognized by cells as intruding pathogens. Foreign objects (cell debris or

bacteria) can initiate inflammatory responses by binding to cell surface receptors (Pivarcsi et al. 2005). IL-8 released as a result of an inflammatory reaction attracts neutrophils and lymphocytes to the inflamed sites to eliminate invading pathogens and halt their spread. Secretion of IL-8 at the ocular surface would induce leukocyte recruiting from superficial tissues like conjunctiva and eyelids; inflammatory response that restricted to the surface areas could minimize deep-tissue damage and help prevent scarring and vision loss. Results of the present study suggested that surfactant proteins are active mediators in an induced inflammatory response by boosting IL-8 in UV or PGN-exposed cells. The findings supported the view that SPs are proinflammatory when harmful objects are present.

Animals such as mouse and rabbits have been traditionally used as a model in corneal research. Although animal studies provide the most convincing results, in vitro studies that use appropriate tools offer time- and money-efficient solutions to studying complicated physiological responses of a biological system; well-designed in vitro studies provide results comparable to that obtained from animal or clinical testing. Nowadays, many countries (in Europe for instance) have banned the use of animals, for example in toxicity studies of skin care products and require the use of an alternative in vitro methods. Hopefully the experimental design in the present study may found to be inspirational to the future researchers.

3.4.2 Effect of SP-depletion on cytokine production

In this study, inhibition of SP expression caused a suppressive effect on IL-8 production in corneal epithelial cells as well as epidermal keratinocytes, suggesting that SP depletion may contribute to immune-suppression manifested by reduced or insufficient cytokine production in an inflammation. Deficiency in immune responses, also called immune-compromise, would greatly increase the chance of an ocular infection. Many factors are known to down-regulate surfactant proteins, including TNF-α (Wispe et al. 1990, Bachurski et al. 1995), reactive oxygen species (ROS), nitric oxide synthase-2 (NOS2) (Baron et al. 2004), 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (Pryhuber et al. 1990), and hydrogen peroxide (H_2O_2) (Merritt et al. 1993).

3.4.3 Effect of exogenous SP on cytokine production

This study demonstrated that surfactant proteins directly associate with IL-8 chemokine modulation in corneal epithelial cells when exposed to UV or PGN that represented as the common environmental threats to ocular health. The study also indicates that up-regulation of SP or supplementation of rhSP may restore an impaired immune function. Known factors up-regulate SPs include keratinocyte growth factor (KGF) (Sugahara et al. 1995), retinoic acid (Bogue et al. 1996), and dexamethasone (Ballard et al. 1996).

Collectin proteins SP-A and SP-D were recently proposed to have dual functions in an inflammatory reaction (Gaidai et al. 2003) and this view has been supported many studies (Vaandrager et al. 2000, Wright. 2005, Takahashi et al. 2006, Kingma et al. 2006, Chaby et al. 2005, Haczku. 2008). According to the theory, SP-A and SP-D exhibit both inflammatory and anti-inflammatory functions depending on if there are foreign objects present. When their Carbohydrate Recognition Domain (CRD) is occupied with cell debris or microorganisms, their collagenous tails bind to calreticulin/ CD91 receptors to stimulate phagocytosis and proinflammatory cytokines. It is well acknowledged that SP-A and SP-D fight against bacteria, fungi and viruses by increasing the permeability of the microbial cell membrane (Wu et al. 2003, McCormack et al. 2003, Restrepo et al. 1999, Ni et al. 2005). Further, SP-A was shown to mediate the phagocytosis of the bacteria by monocytes by binding to both Staphylococcus aureus bacteria and the C1q receptor on moncytes (Geertsma et al. 1994). On the other hand, during steady-state conditions the CRD of SP-A and SP-D binds to Toll-like receptor 2 (TLR 2) or signal inhibitory regulatory protein alpha (SIRPα), this interaction induces

downstream inhibition of mitogen-activated protein kinase (MAPK) mediated NF-$_{\kappa}$B transcription factor, leading to the inhibition of proinflammatory cytokines (Takahashi et al. 2006, Sato et al. 2003). SP-A and SP-D have a general protective role as they keep inflammation in check under normal circumstances and become fully engaged in an inflammation when cells sense a threat. The present study partially supported the theory. However, direct evidence is still lacking regarding the anti-inflammatory activities of SP. The experimental challenge is the extremely low production of cytokines under non-stimulated conditions. An interesting observation in the present study is that SP-A and SP-D have rather different effects on IL-8 production, suggesting that these proteins may be functionally complementary. Despite their structural similarity, SP-A and SP-D are quite different in biochemical properties which would likely confer distinctive functional properties (Crouch. 1998).

Also possessing antimicrobial capabilities, surfactant proteins could potentially provide therapeutic solutions to treating non-healing or chronic wounds because these “natural antibiotics”- as opposed to conventional antibiotics, would not only prevent the growth of drug-resistant microbes but also expedite the wound healing process by promoting immune responses to fight against infectious agents.

3.4.4 Effect of external stimulus on SP production

This is the first study to show the stimulation of SP production by UV, LPS and PGN. In one study, UV was shown to associate with SP-A recruiting from non-irradiated skin to irradiated skin and SP-A was found to bound with apoptotic keratinocytes and facilitate the uptake of these cells by monocyte-derived dendritic cells (Lokitz et al. 2005). In another study, it was shown that SP-D gene in mammalian epidermal cells contain binding sites for AP-1 family, and other transcription factors that are activated by JNK, stress-activated MAP kinase (He et al. 2000). UV and PGN as the most common environment insults to the eye are known to induce inflammation via MAPK signaling pathways (Kumar et al. 2004), it is plausible that induction of SP-B and SP-C, as was found in the present study, may also relate to MAPK pathway. Further investigation is required.

3.4.5 Internalization of heat-killed bacterial by epithelial cells

The present study also demonstrated invasion of heat-killed S. aureus bacteria in corneal epithelial cells; the invasion occurs within 3 hours after the bacterial challenge. The adherence and binding of the bacterial to epithelial cells are believed to depend on fibronectin-binding protein (FnBp) expressed on the surface of the bacteria (Dziewanowska et al. 1999). Fibronectin acts as a bridging molecule between FnBps on the bacteria and $\alpha_5\beta_1$ integrin on the host cell. $\alpha_5\beta_1$ integrin is known to be expressed in the human corneal epithelium (Virtanen et al. 1999). Interaction of the bacteria and epithelial cells activates intracellular signaling cascades leading to actin polymerization and formation of endocytic vesicles.

3.4.6 Future prospects

Cytokine modulation by hydrophobic SP-B and SP-C is yet an unexplored territory. This is the first study that links SP-B and SP-C to cytokine modulation in corneal epithelium. SP-B (native or synthetic) has been shown to have anti-microbial activity by selectively lysing bacterial membranes (Ryan et al. 2005). SP-C, but not SP-B has been demonstrated to interact with membrane bound CD14, a receptor for lipoploysaccharide (LPS) suggesting it may have immunological role during inflammatory processes (Augusto et al. 2003, Mulugeta et al. 2006). However experimental evidence linking SP-C to cytokine regulation is still lacking. Findings of this study expand the roles of these hydrophobic surfactant proteins from what have been traditionally thought to be only associated with surface tension reduction in lung alveoli. Future studies would be focused on the mechanisms governing SP-B and SP-C in cytokine regulations.

This study supported the view that surfactant proteins at the ocular surface proactively participate in immunological responses triggered by danger signals (e.g. microbial or apoptotic cell debris). They act to eliminate bacteria and other objects detrimental to the ocular health via several independent approaches of which the effect can be direct or indirect. Secretion of surfactant proteins into the tear fluid further potentiates their strength and expands their scope of action, contributing to prompt initiation and quick resolution of an inflammation reaction thus minimizing the scar tissue formation. Presence of surfactant proteins in tear fluid offers another evidence that tear fluid serves not only to lubricate the eye and retain its moisture; but also to maintain and regulate the

corneal and conjunctival epithelial functions by providing biological active substances to the surface regions.

Unlike most tissues in the body, changes of cornea due to aging are mostly without symptoms; often times do not affect vision, and hence do not require treatments. However with age, corneal epithelium undergoes some deterioration resulting in a breakdown of epithelial barrier, which may explain why aged cornea is more susceptible to infection. A reduced ability of corneal cells to upregulate adhesion molecules and a reduced phagocytic ability of reactive neutrophils in response to reaction also occur with aging, this could impair the ability to eliminate a bacterial infection. Surfactant proteins, as essential components in ocular host defense and barrier functions, may have therapeutic implications in relieving ocular disorders such as infections in aged or immunocompromised patients.

3.5 Summary and conclusions

This study shows that in ocular epithelial cells, surfactant proteins promote inflammatory cytokine productions in response to external stimulus such as UV irradiation and bacterial challenge.

SP-A, SP-B, SP-C and SP-D expressed in human corneal epithelial cells and epidermal keratinocytes were found to augment cytokine and chemokine production in response to UV and PGN, two common environmental insults to the eye. Cells depleted with surfactant proteins shown to have reduced ability in producing inflammatory cytokines, whereas exogenous surfactant proteins were observed to be able to recover the impaired immune responses in cells of which surfactant protein expression was suppressed.

The stimulatory effect of surfactant proteins on cytokine production were found to vary among different types of proteins, SP-A appeared to be more potent than SP-D. The findings that SP-B and SP-C also modulate immune reactions are rather intriguing because these hydrophobic proteins were traditionally thought to only involve in surface tension reduction and play a minor role in inflammation.

UV, LPS and PGN as the potent inflammatory inducers were found to also stimulate surfactant proteins in cells, supported the view that surfactant proteins are active participants in inflammation reactions. Combined with their activities previous known, this study supports the view that all types of surfactant proteins are more or less involved in biophysical and biological aspects of the ocular surface, ranging from tear film stabilizing and distribution, to bacterial elimination, immunological reactions and wound healing.

4 SP in modifying surface tensions of ophthalmic materials

4.1 Silicone lens and artificial tear fluid

4.1.1 Silicone lens

As contact lenses sit on the eye, its anterior surface is covered by the lipid- aqueous layer of tear film; and the posterior surface is in contact with the base mucous-aqueous layer of the tear film (Figure 17). Silicon that used as contact lens material is hydrophobic in nature, and requires some degree of chemical alteration to allow for adequate compatibility with the corneal epithelium on both the posterior and anterior lens surfaces. Failure to create water-loving surfaces can result in lens dehydration, poor wettability, and increased lens friction. These factors can result in clinical manifestations of ocular dryness and discomfort.

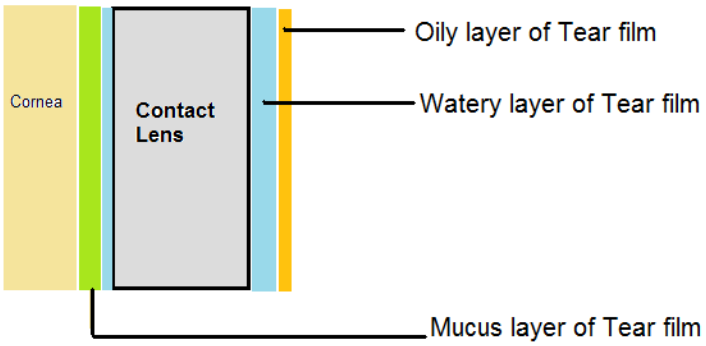


Fig. 17 Contact lens and pre-/ post-lens tear films

Take silicone as an example, the challenge for contact lens manufacturing is to turn the hydrophobic surface into one that will attract and spread the tear film. Some manufactures utilize lens surface treatment such as surface plasma treatment which results in a newly formed hydrophilic surface. Others use additive agents (e.g. wetting agent polyvinyl-pyrrolidone PVP) that become an inherent part of the lens matrix,

thus eliminating the problem of removal of the surface coating material through lens wear or rubbing. Contact lens with improved surface wettability provides better comfort and less contact lens-induced dry eye. Ocular surfactant proteins, particularly hydrophilic SP-A and SP-D may be deposited onto the surfaces of contact lens to enhance lens hydrophilicity. Surfactant proteins possess anti-microbial activities; their slow release from the lens surface may offer additional protections to the eye.

4.1.2 Artificial tear fluid

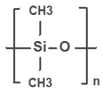
Artificial tear fluid provides lubricating effect, enhances wettability of the corneal surface, and promotes the retention of ocular surface moisture. Artificial tear products offer relief for the dry eye. Dry eye is a disorder of the tear film due to tear deficiency or excessive tear evaporation which causes damage to the exposed surface of the eye and is associated with symptoms of eye discomfort.

Designing artificial tears for the treatment of dry eye is based on the understanding of certain properties of natural human tears. Ideal artificial tears should adequately replace the natural tears and its functions. Key physical properties involved in the function of tears are surface tension and viscosity, both of which are thought to be important in spreading and maintenance of the tear film. The tear fluid is what is referred to as a “non-Newtonian” fluid, which means that the viscosity depends on the shear rate (the amount of movement in the fluid). The greater the shear rate, the lower the viscosity (shear-thinning) (Tiffany. 1991). Such property has advantages in that it resists gravitational drainage at low shears (eye open) while avoids viscous dragging and epithelial damage at high shears (blinking). In another words, the viscosity must be high enough to maintain

a continuous layer of the tear film that covers the exposed area of the ocular surface. Meanwhile, it must also allow the eyelids to rapidly move across the ocular surface without necessary dragging of the epithelium, which would be painful and considerably slow down the blinking movement of the eyelids.

An artificial tear fluid is commonly composed of electrolytes to simulate the salt content of the natural tear fluid, a nonionic surfactant to lower the surface tension of the compositions and enhance the spreading of the compositions over the surface of the cornea, and antimicrobial preservatives. Most preservative, however, are toxic to the epithelial cells and disrupt the lipid layer of the tear film (Holly et al. 1978), thus making the tear film even more unstable and can aggravate the damage caused by the disease in the ocular surface. Other adverse effects of preservatives include disruption of intracellular desmosomes (Bernal et al. 1991), increasing the corneal permeability (Ramselaar et al. 1988), decreasing the activity of Lysozyme in the tear fluid (Haeringen et al. 1993), and sensitization with risk for ocular allergy (Fischer et al. 1972). Surfactant proteins possess anti-microbial activities and may become promising replacement to harsh preservatives currently used in artificial tear products.

4.2 Objectives

PDMS ( , polydimethysiloxane) are often referred to as silicones. Silicone polymers can be easily transformed into elastomers by a way of cross-linking that allows the formation of chemical bonds between adjacent chains. Silicone elastomers have been widely used in biomedical applications such as medical-grade tubing, transdermal drug delivery patches, implanted prostheses and contact lenses. Silicone-based materials are considered to be biocompatible (i.e. lack of toxicity and minimal tissue response) and bio-durable (i.e. thermal and chemical stable) attributed to their unique material properties such as high oxygen permeability, flexibility (low glass transition temperature T_g typically less than 120^{o}C) and stability (chemically inert). However silicone materials are hydrophobic in nature due to their non-polar/ hydrophobic methyl (-CH_3) side groups pointing towards the surfaces, and in many cases would require some degree of chemical alteration to allow for adequate compatibility with the host tissue (i.e. corneal surface).

Wettability of lenses allows tear film to spread evenly, consistently and completely across the lens surface. The present study sets out to investigate the feasibility of using SP in ophthalmic biomaterials, with specific goals to determine if surface deposition of SP can enhance silicone surface wettability. It would be also interesting to evaluate the effect of SP on the surface tension of an existing artificial tear product. SP also possess anti-microbial activities; their release from the lens surface presumably would offer additional protections to the eye. Besides, anti-bacterial SP may become promising replacement to harsh preservatives currently used in artificial tear products.

4.4 Materials and Methods

4.4.1 Silicone rubber

Transparent and clear silicone rubber sheet with thickness of 0.02 inch was purchased from Rubber Sheet Roll (Shippensburg, PA). The silicone sheet has plastic backings on both sides that are peeled off upon testing. To prepare silicone samples, the sheet was cut into small pieces of 0.5 in x 0.5 in and mounted onto a glass slide. The surfaces of the silicone sheet are considered to be flat, smooth, homogeneous and non-swelling.

4.4.2 Artificial tear fluid

OptiZen® is an over-the-counter product claims to relieve eye discomforts experienced by computer users. It contains polysorbate 80.

For comparison purposes, SP were added to either the eye drop or 100 ug/ml phosphatydylcholine (PL) and the surface tension reduction effects of SP were evaluated.

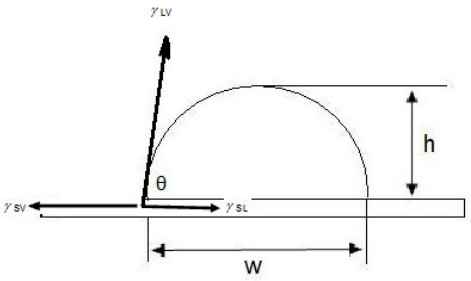
4.4.3 Surfactant protein adsorption on the silicone surface

Recombinant surfactant protein D (1920-SP-050, R&D systems, Minneapolis, MN) as well as recombinant SP-B (Cusabio Biotech, Wuhan, China) were dissolved in 0.01M PBS solution to 10 ug/ml and 1 ug/ml respectively. Under ambient conditions, 150ul of the solution (with or without SP) was placed onto a clean surface of the silicone sample and was subsequently kept in a closed container for 6 hours at room temperature. After incubation, silicone samples were rinsed twice with Millipore-Q water to remove excessive or loosely adsorbed proteins.

Silicone samples were then air dried for approximately 15 minutes before any further testing.

4.4.4 Contact angle measurement

Hydrophobicity of the silicone surface was evaluated by measuring the contact angle formed between water drops and the surface of the SP-B, or SP-D coated silicone by using a CAM 200 Optical Contact Angle Meter (KSV instruments Ltd., Helsinki, Finland). For this purpose, 2 ul of water droplets were mounted on the coated or non-coated surface with an Eppendorf micropipet. Images of the water drop at a rate of 1 frame per 20 seconds were recorded and stored. To evaluate the effect of SP on surface tensions of artificial tear fluids, contact angles of 10ul droplets of commercialized artificial tear (with or without the addition of SP) were also analyzed. The definitions of drop contact angle, base diameter, and height are described below. Using the CAM200 software a curve was fitted to the drop parameter using the Young-Laplace equation. The contact angle was then determined from the slope of the contour line at the three-phase boundary point.



The equilibrium at a solid (S) and vapor (V) triple line is described by Young's equation

$$\gamma_{SV} = \gamma_{SL} + \gamma_{LV} \cos\theta_0 \quad \text{eq1}$$

where, γ_{SV}, γ_{SL} and γ_{LV} represent surface tensions for the solid/vapor, solid/liquid and liquid/vapor interfaces and θ_0 is the equilibrium contact

angle between the tangent planes to the S/L and L/V boundaries at the three phase line or triple line. The contact angle as given by Young's equation is a static and equilibrium angle. However, during its motion toward an equilibrium shape, a liquid droplet spans a range of dynamic contact angles. The volume V(t) and the surface area A(t) of a water drop were calculated from eq. 2 and eq. 3 by assuming the spherical cap model. Therefore, evaporation flux J(t) that is defined as the mass loss per unit time per unit surface area of the water drops can be derived (eq. 4). Due to the high scattering of evaporation flux data, evaporation rates (mass loss over unit time) were calculated to evaluate the evaporation processes of the water drops on silicone surfaces.

$$V(t) = \frac{\pi W^3 \left(2 - 3\cos\theta + \cos^3\theta\right)}{24\sin^3\theta} \qquad \text{eq. 2}$$

$$A(t) = \frac{\pi W^2}{2(1+\cos\theta)} \qquad \text{eq. 3}$$

$$J(t) = \frac{V_{t-1} - V_t}{A_{t-1}} \qquad \text{eq. 4}$$

At least three droplets were observed during their evaporation on each sample and showed good statistical consistency. The evaporation curves presented here were chosen as typical for each sample.

4.4.4 Topology imaging by Atomic Force Microscopy (AFM)

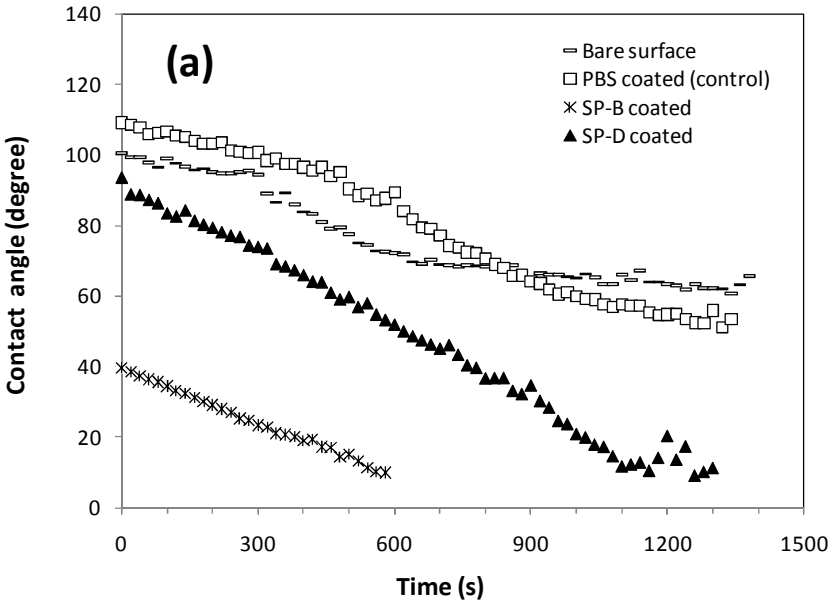
The silicone samples were mounted on glass slides and imaged with a Nanoscope IIIa instrument (Digital Instruments, Santa Narbara, CA) using tapping mode in air using tips (PPP-NCHR, Nanosensor) with spring constants of 42 N/m and resonance frequencies of 330 kHz. Tapping mode AFM is a technique in which the imaging probe is vertically oscillated near the resonant frequency of the cantilever. Electro-mechanical feedback maintains the oscillation at constant amplitude during scanning. The tip intermittently touches or "taps" the surface. The main advantage of tapping mode is the elimination of lateral shearing force present in the contact mode, thus reducing the possibility of smearing the protein molecules on the surface. Images were collected at a scan rate of 1.0 Hz and a scan size of 10 um. Silicone sample coated with PBS containing no proteins was served as the control.

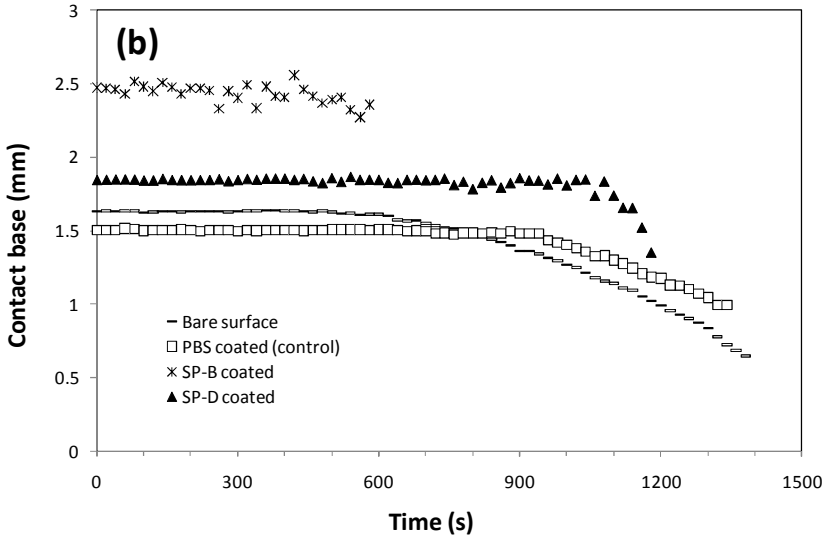
4.5 Results and discussion

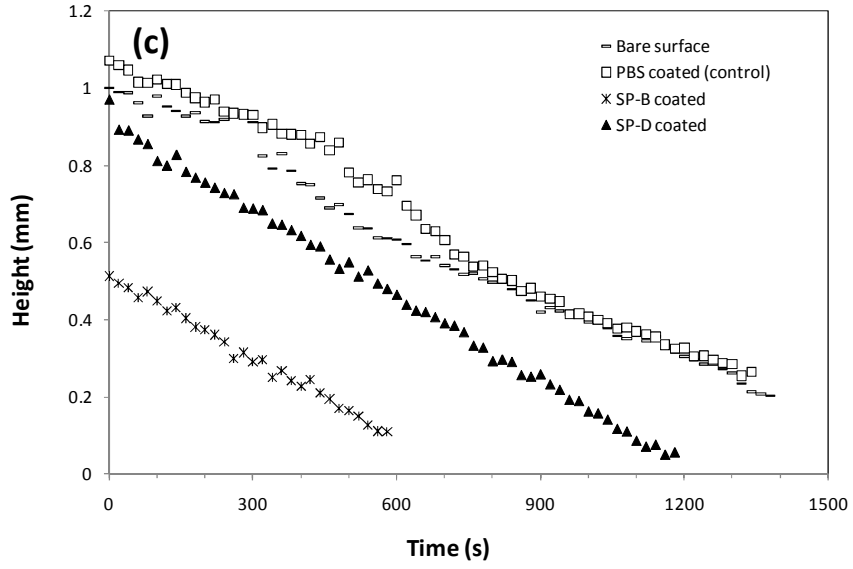
4.5.1 Wetting behavior of SP-coated silicone surfaces

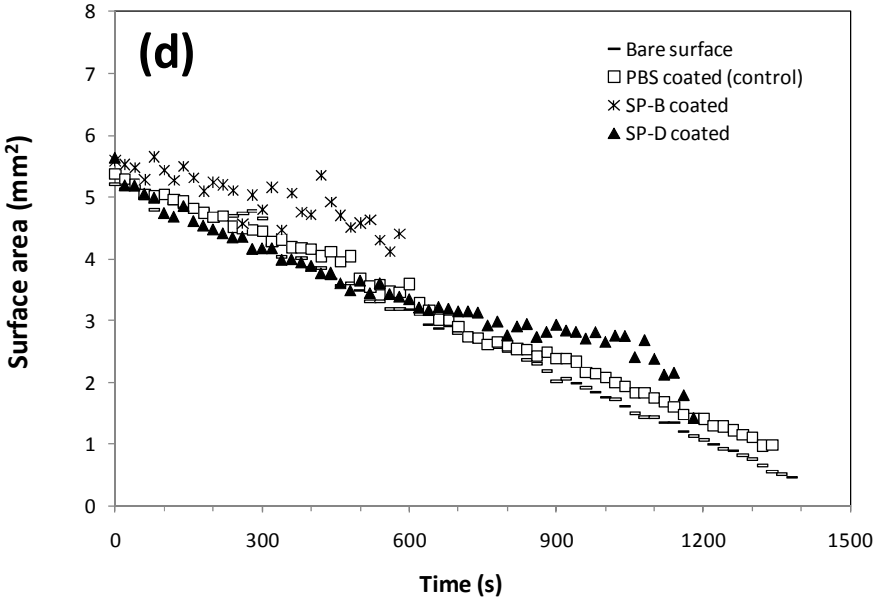
Contact angles, contact bases and heights of the water droplets on the coated and non-coated PDMS surfaces were plotted versus time (Figure 18). The water drop contact angles on SP coated surfaces were lower than uncoated surface with contact bases remained constant over the entire period of the experiment. A general consensus is that with initial contact angles less than 90^{o} decreasing in contact angle with constant contact base is due to drop evaporation (Erbil et al, 2002). Apparently this theory is also applicable to the uncoated silicone which was observed to exhibit hydrophobic character during the first two minutes.

Evaporation of water drops on the silicone surfaces (SP- coated and non-coated) were observed to evolve in two distinct stages: in the first stage, the base of the drop stayed constant while the contact angle and the height of the water drop decreased linearly and simultaneously. This stage lasted for a long period of time during the evaporation. At the end of this stage, the base abruptly decreased but the angle and the height of the drop remained relatively fixed. This stage was rather short and lasted until the drop disappeared. Due to the high scattering of evaporation flux data, evaporation rates (mass loss over unit time) were calculated to evaluate the evaporation processes of the water drops on silicone surfaces. It turned out that water drops on the SP-coated surfaces evaporated faster than those on the non-coated surfaces as their slopes of the linear fit of the volume plots were determined to be 0.00116, 0.00130, and 0.00132 mg/second for the control, SP-B and SP-D coated surfaces.


(a)
Bare surface
PBS coated (control)
SP-B coated
SP-D coated
Contact angle (degree)
Time (s)
0
20
40
60
80
100
120
140
0
300
600
900
1200
1500


(b)
Bare surface
PBS coated (control)
SP-B coated
SP-D coated
Contact base (mm)
Time (s)
0
0.5
1
1.5
2
2.5
3
0
300
600
900
1200
1500


(c)
Bare surface
PBS coated (control)
SP-B coated
SP-D coated
Height (mm)
Time (s)
0
0.2
0.4
0.6
0.8
1
1.2
0
300
600
900
1200
1500


(d)
Bare surface
PBS coated (control)
SP-B coated
SP-D coated
Surface area (mm²)
Time (s)
0
1
2
3
4
5
6
7
8
0
300
600
900
1200
1500

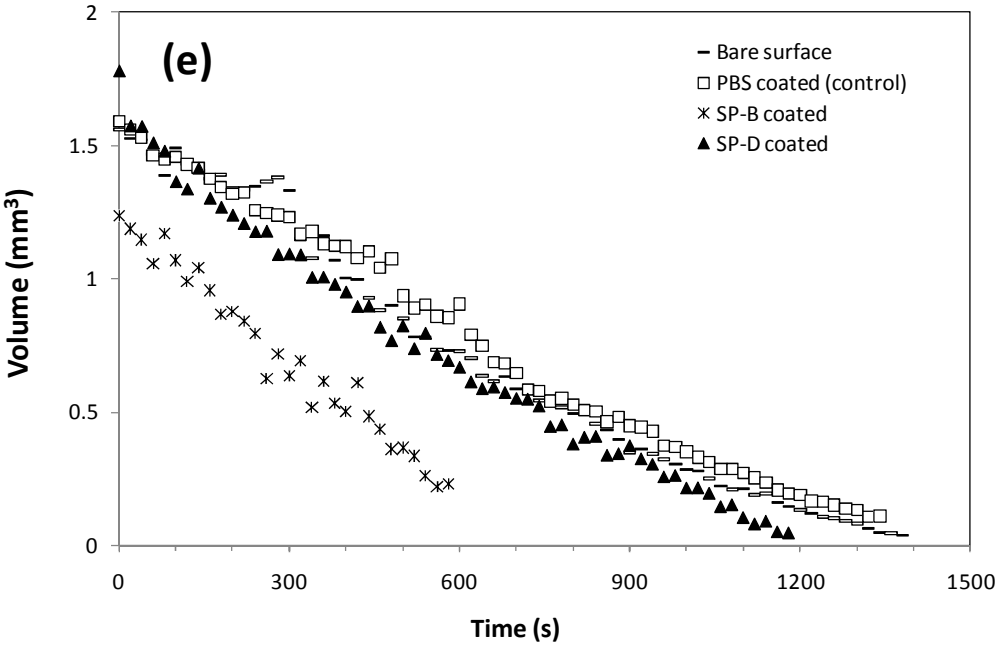


Fig. 18 (a) Contact angle θ, (b) contact base diameter W, (c) drop height h, (d) surface area A, and (e) drop volume V for 2 µl drops of water on SP coated and non-coated silicone surfaces.

Since wetting is governed by molecular interaction in the outermost surface layer of a few angstroms, the forces dictating the wetting behavior originate from the outermost surface groups. The molecules tend to arrange themselves in the surface layer in such a way that their interfacial tension with the surrounding phase is minimized. Take SP-D as an example, in an aqueous solution SP-D proteins expose their hydrophilic moieties (collagen-like domain) at their interface with the water and may tend to concentrate at the water-air interface, where they orient part of their hydrophobic moieties (carbohydrate recognition domain) towards the air. Consequently, the air-dried particles of solid SP-D would have an outward oriented hydrophobic layer which explains that the protein coating

initially appeared to be hydrophobic and then turned hydrophilic when re-dissolved in water.

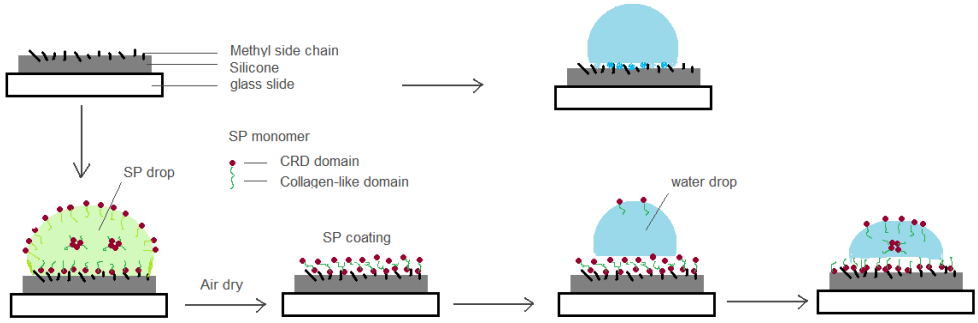
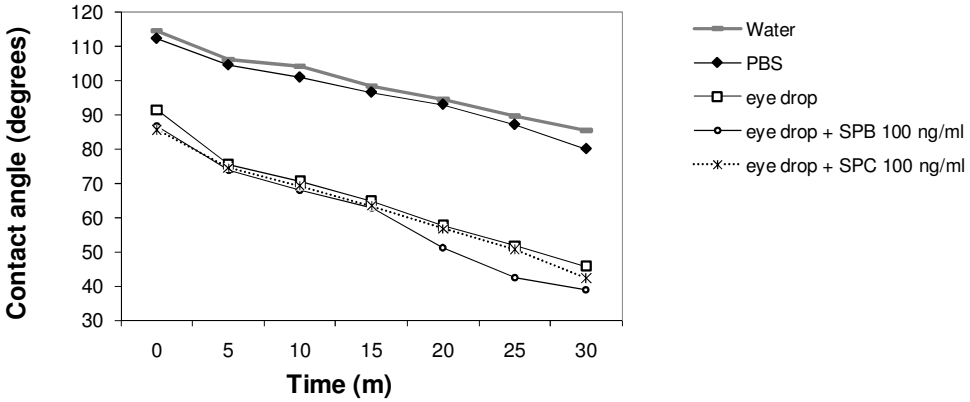


Fig. 19 Effect of the coating and wetting processes on protein arrangement on the silicone surface

4.5.2 Effect of SP on surface tensions of surfactant liquids

Artificial tear samples exhibited much lower surface tension compared with water or PBS buffer solution. Addition of SP-B and SP-C appeared to have caused a further reduction in surface tension of the tested artificial tear fluid, manifested by decreased contact angles formed by the solution with the silicone surface. It was also noted that SP-B had a more dramatic effect than SP-C and SP-D on reducing surface tensions of the phosphatydylcholine solution.

a) Commercial eye drop (OptiZen®)



b) 100 ug/ml phosphatydylcholine (PL) in 1% EtOH solution

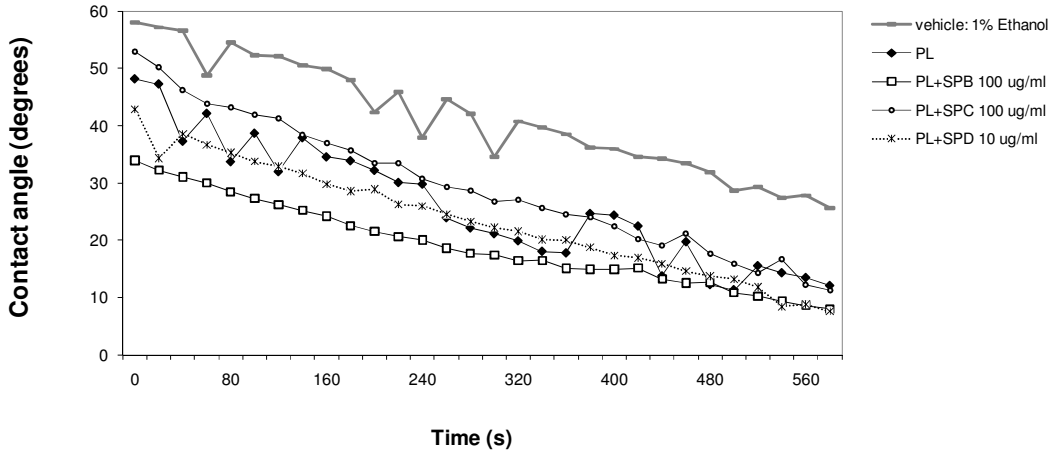


Fig. 20 Contact angle vs. time plots for eye drop (a) and phophatydylcholine (b) containing SP-B, SP-C or SP-D

4.5.4 Surface topology by Atomic Force Microscopy (AFM)

AFM images of the SP-D coated and non-coated silicone surfaces were characterized in air by AFM with the tapping mode. The measured AFM images were analyzed for surface roughness. The section analysis was conducted to depict sectional profiles of the adsorbed protein molecules. Quantitative measurements of the root mean square (RMS) surface roughness were determined using 10µm x 10µm scans. The RMS roughness is defined as the height fluctuation in a given area:

$$Rms = \sqrt{\sum_{j=1}^{N}\sum_{i=1}^{N}\frac{\left(Z_{ave} - Z_{ij}\right)^2}{N^2}} \qquad \text{eq. 5}$$

where, Z_{ave} and N are the average values and the number (512) of Z_{ij} (surface height), respectively. The RMS roughness measurements for three uncoated and coated silicone were 17.6 ± 0.26 nm and 12.2 ± 0.31 nm respectively. SP-D coated silicone appeared to be slightly smoother than the uncoated.

The AFM images (Figure 22) revealed that the surfaces exposed to 10 ug/ml of SP-D were covered with many small compact protein clusters. Because of the dominant surface functional group $–CH_3$, protein adsorption on silicone is mainly through hydrophobic interaction. One would assume that adsorbed SP-D can take either the “end-on” position with the characteristic height of 114 nm (length of two adjoined collagen arms) or the “side-on” position with the characteristic height of 9 nm (diameter of globular CRD domain) for a monolayer of proteins (Figure 5). Sectional analysis revealed that thickness of the protein coating was 99.6 ± 0.03 nm, corresponds to the height of a SP-D dodecamer/ multimer molecule in an “end-on” position (≤ 114 nm depending on the angle

formed between extended collagen arms). This observation suggested that SP-D dodecamers or higher-ordered multimers may interact with silicone surface methyl groups through their CRD domains with the rigid collagen-like arms sticking towards the air (Figure 21b). Although protein molecules may assume the “side-on” position with multiple layers of molecules stacking on top of each other (Figure 21a), it appeared that single-layered protein molecules in the “end-on” position would allow for hydrophobic groups that exposing to the air to be more densely packed, and thus is considered more energetically preferable than the multiple layers of proteins in the “side-in” position.

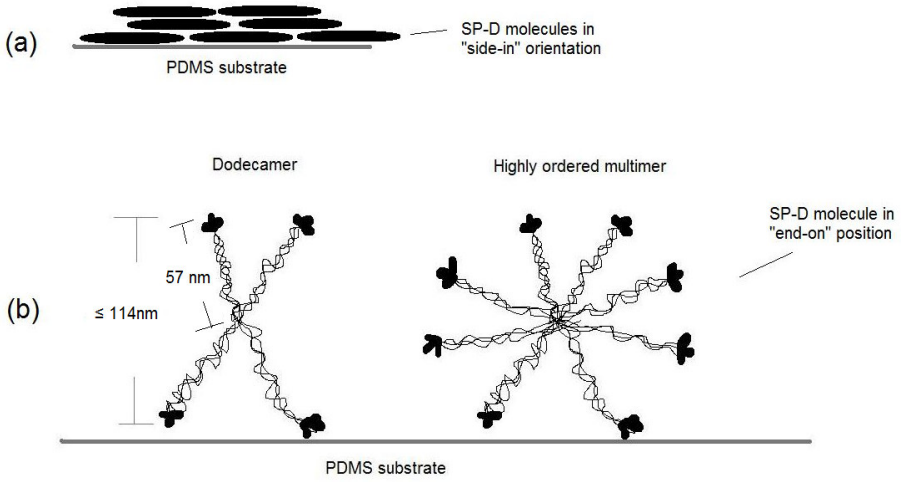


Fig. 21 Proposed orientation of SP-D molecules on the silicone surface: (a) the “side-in” position where multiple layers of molecules are stacked on top of each other; (b) the “end-on” position where a single layer of molecules are adsorbed on the surface with CRD exposing to the air

Uncoated silicone surface SP-D coated silicone surface

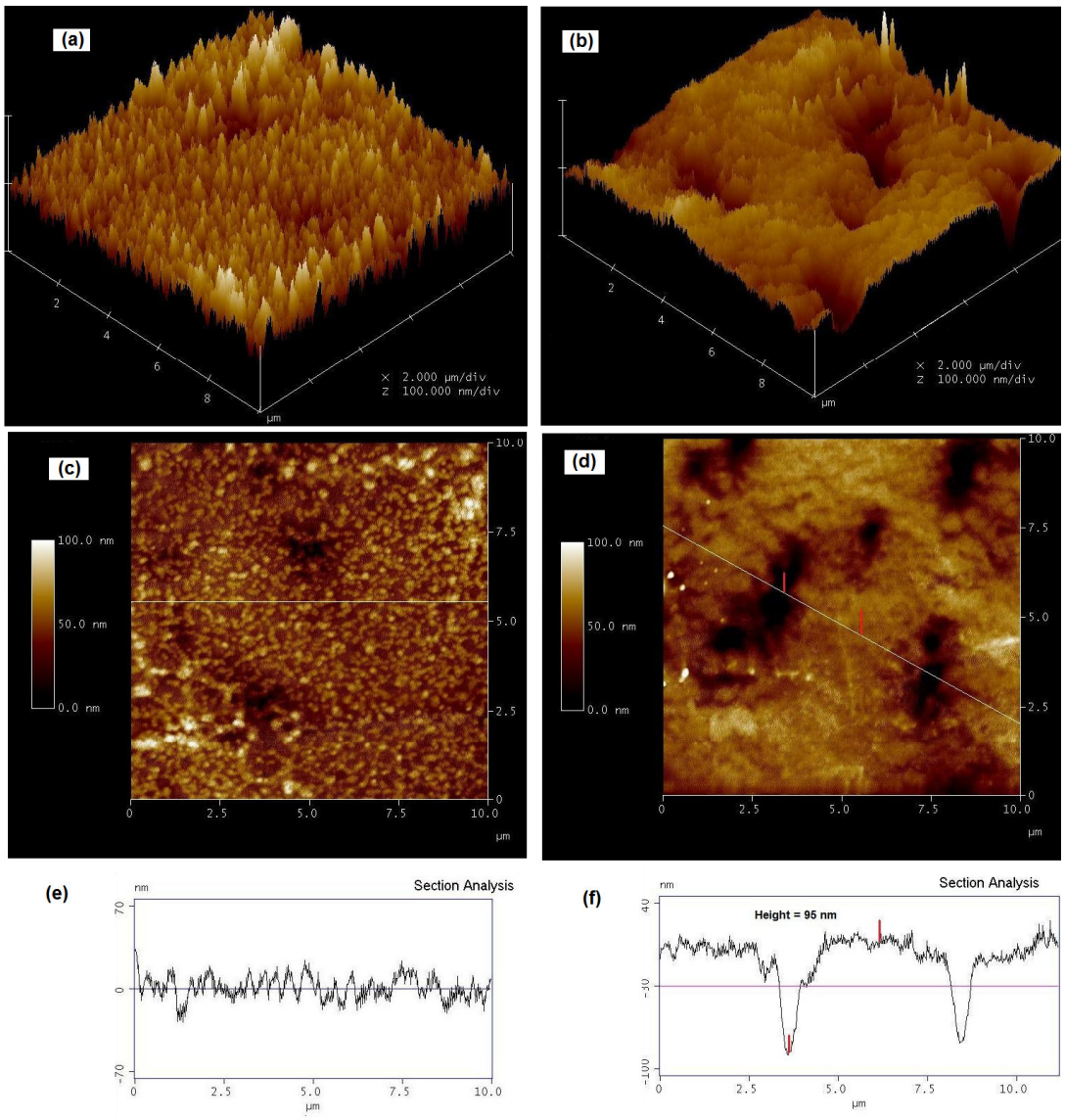


Fig. 22 Tapping mode AFM images of SP-D coated and uncoated silicone samples: height map and top view of the control sample (a, c) and the coated sample (b, d); sectional analysis of the control sample (e) and the coated sample (f)

Conclusion

The present study demonstrated that silicone surfaces adsorbed with surfactant proteins had improved wettability evidenced by contact angle measurements. AFM image analysis indicated that SP-D coated silicone surfaces may be covered with a single layer of protein molecules. It is speculated that surfactant protein adsorption is a simple and effective approach to improving surface wettability of silicone-based biomaterials. Additionally, inclusion of SP appeared to further decrease the surface tensions of the artificial tear drops. Taken together, SP is speculated to be useful in improving ophthalmic biomaterial properties. Future work may be focused on refining the methods of surface treatment by either physical adsorption or chemical bonding. Biological effects as a result of the released protein from the modified surfaces will be further evaluated.

References

Araki-Sasaki, K., Ohashi, Y., Sasabe, T., Hayashi, K., Watanabe, H., Tano, Y., Handa, H. 1995. An SV-40-immortalized human corneal epithelial cell line and its characterization. Invest Ophthalmol Vis Sci. 36: 614-21.

Augusto, L.A., Synguelakis, M., Johansson, J., Pedron, T., Girard, R., Chaby, R. 2003. Interaction of pulmonary surfactant protein C with CD14 and lipopolysaccharide. Infect Immun. 71 (1): 61-67

Azghani, A.O., Baker, J.W., Shetty, S., Miller, E.J., Bhat, G.J. 2002. Pseudomonas aeruginosa elastase stimulates ERK signaling pathway and enhances IL-8 production by alveolar epithelial cells in culture. Inflamm. Res. 51: 506-510.

Bachurski, C.J., Pryhuber, G.S., Glasser, S.W., Kelly, S.E., Whitsett, J.A. 1995. Tumor necrosis factor-α inhibits surfactant protein C gene transcription. J. Bio. Chem. 270 (33): 19402-7.

Ballard, P.L., Ertsey, R., Gonzales, L.W., Gonzales, J. 1996. Transcriptional regulation of human pulmonary surfactant proteins SP-B and SP-C by glucocorticoids. Am J Respir Cell Mol Biol. 14 (6): 599-607.

Bals, R., Hiemstra, P.S. 2004. Innate immunity in the lung: how epithelial cells fight against respiratory pathogens. Eur Respir J. 23:327–333.

Barker, J.N., Jones, M.L., Mitra, R.S., Crockett-Torabe, E., Fantone, J.C., Kunkel, S.L., Warren, J.S., Dixit, V.M., Nickoloff, B.J. 1991. Modulation of keratinocyte-derived interleukin-8 is chemotactic for neutrophils and T lymphocytes. Am J Pathol. 139 (4): 869-76.

Baron, R.M., Carvajal, I.M., Fredenburgh, L.E., Liu, X., Porrata, Y., Cullivan, M.L., Haley, K.J., Sonna, L.A., De Sanctis, G.T., Ingenito, E.P., Perrella, M.A. 2004. Nitric oxide synthase-2 down-regulates surfactant protein-B expression and enhances endotoxin induced lung injury in mice. FASEB J. 18 (11): 1276-8.

Bender, K., Blattner, C., Knebel, A., Iordanov, M., Herrlich, P., Rahmsdorf, H.J. 1997. UV induced signal transduction. [Review]. J Photochem Photobiol B Biol. 37: 1-17.

Benko, S., Tozser, J., Miklossy, G., Varga, A., Kadas, J., Csutak, A., Berta, A., Rajnavolgyi, E. 2008. Constitutive and UV-B modulated transcription of Nod-like receptors and their functional partners in human corneal epithelial cells. Mol Vis. 14: 1575-83.

Bernal, D.L., Ubels, J.L. 1991. Quantitative evaluation of the corneal epithelial barrier: effect of artificial tears and preservatives. Curr Eye Res. 10(7): 645-56.

Blau, H., Riklis, S., Kravtsov, V., Kalina, M. 1994. Secretion of cytokines by rat alveolar epithelial cells: possible regulatory role for SP-A. Am J Physiol. 266: L148-L155.

Bogue, C.W., Jacobs, H.C., Dynia D.W., Wilson, C.M., Gross, I. 1996. Retinoic acid increases surfactant protein mRNA in fetal rat lung in culture. Am J Physiol. 271 (5 Pt 1): L862-868.

Borron, P., McIntosh, J.C., Korfhagen, T.R., Whitsett, J.A., Taylor, J., Wright, J.R. 2000. Surfactant protein-A inhibits LPS-induced cytokine and nitric oxide production in vivo. Am J Physiol (Lung Cell Mol Physiol). 278: L840-7.

Bräuer L, Johl M, Börgermann J, Pleyer U, Tsokos M, Paulsen FP. 2007. Detection and localization of the hydrophobic surfactant proteins B and C in human tear fluid and the human lacrimal system. Curr Eye Res. 32(11):931-8.

Bräuer L, Kindler C, Jäger K, Sel S, Nölle B, Pleyer U, Ochs M, Paulsen FP. 2007. Detection of surfactant proteins A and D in human tear fluid and the human lacrimal system. Invest Ophthalmol Vis Sci. 48(9):3945-53.

Brauer, L., Johl, M., Paulsen, F.P., Borgermann, J., Pleyer, U., Tsokos, M. 2007. Detection and localization of hydrophobic surfactant protein B and C in human tear fluid and the human lacrimal system. Curr Eye Res. 32: 931-38.

Bruhn, H.Riekens, B., Berninghausen, O., Leippe, M. 2003. Amoebapores and NK-lysin, members of a class of structurally distinct antimicrobial and cytolytic peptides from protozoa and mammals: a comparative functional analysis. Biochem J. 375: 737-44.

Brummer, E., Stevens, D.A. 2009. Collectins and fungal pathogens: role of surfactant proteins and mannose binding lectin in host resistance. Med Mycol. 9999:1-13.

Chaby, R., Verdugo, I.G., Espinassous, Q., Augusto, L.A. 2005. Interaction between LPS and lung surfactant proteins. J Endotoxin Res. 11(3): 181-5.

Cheng, G., Ueda, T., Numao, T., Kuroki, Y., Nakajima, H., Fukushima, Y., Motojima, S., Fukuda, T. 2000. Increased levels of surfactant protein A and D in bronchoalveolar lavage fluids in patients with bronchial asthma. Eur Respir J. 16: 831-5.

Crouch E, Persson A, Chang D, Heuser J. 1994. Molecular structure of pulmonary surfactant protein D (SP-D).J Biol Chem. 269(25):17311-9.

Crouch, E.C. 1998. Structure, biologic properties, and expression of surfactant protein D (SP-D). Biochem. Biophy. Acta. 1408: 278-289.

Cubitt, C., Tang, Q., Monteiro, C.A., Lausch, R.N., Oakes, J.E. 1993. IL-8 gene expression in cultures of human corneal epithelial cells and keratinocytes. Invest Ophthalmol Vis Sci. 34: 3199-3206.

Ding, J., Takamoto, D.Y., von Nahmen, A., Lipp, M.M., Lee, K.Y.C., Waring, A.J., Zasadzinski, J.A. 2001. Effects of lung surfactants, SP-B and SP-C, and palmitic acid on monolayer stability. Biophys J. 80:2262-72.

Dziewanowska, K., Patti, J.M., Deobald, C.F., Bayles, K.W., Trumble, W.R., Bohach, G.A. 1999. Fibronectin binding protein and host cell tyrosine kinase are required for internalization of Staphylococcus aureus by epithelial cells. Infect. Immun. 67: 4673-8

Elner, V.M., Strieter, R.M., Pavilack, M.A., Elner, S.G., Remick, D.G., Danforth, J.M., Kunkel, S.L. 1991. Human corneal interleukin-8: IL-1 and

TNF-induced gene expression and secretion. Am J Pathol. 139 (5) 977-88.

Ernst, W.A., Thoma-Uszynski, S., Teitebaum, P., Ko, C., Hanson, D.A., Clayberger, C., Krensky, A.M., Leippe, M., Bloom, B.R., Ganz, T., Modlin, R.L. 2000. Granulysin, a T-cell product, kills bacteria by altering membrane permeability. J Immunol. 165: 7102-8.

Feugate, J.E., Li, Q., Wong, L., Martins-Green, M. 2002. The cxc chemokine cCAF stimulates differentiation of fibroblasts into myofibroblasts and accelerates wound closure. J Cell Biol. 156(1):161-72.

Fischer, A.A., Stillman, M.A. 1972. Allergic contact sensitivity to benzalkonium chloride. Arch Derm. 106: 169-71.

Gardai, S.J., Xiao, Y.Q., Dickinson, M., Nick, J.A., Voelker, D.R., Greene, K.E., Henson, P.M. 2003. By Binding SIRPα or Calreticulin/CD91, Lung Collectins Act as Dual Function Surveillance Molecules to Suppress or Enhance Inflammation. Cell. 115(1):13-23.

Geertsma, M.F., Nibbering, P.H., Haagsman, H.P., Daha, M.R., van Furth, R. 1994. Binding of surfactant protein A to C1q receptors mediates phagocytosis of Straphylococcus aureus by monocytes. Am J Physiol. 267 (5 pt 1): L578-584.

Glasser, S.W., Detmer, E.A., Ikegami, M., Na, C.L., Stahlman, M.T., Whitsett, J.A. 2003. Pneumonitis and emphysema in SP-C gene targeted mice. J. Biol. Chem. 278: 14291-8

Gold, J.A., Hoshino, Y., Tanaka, N., Rom, W.N., Raju, B., Condos, R., Weiden, M.D. 2004. Surfactant protein A modulates the inflammatory response in macrophages during tuberculosis. Infect Immun. 72(2): 645-50.

Greene, K.E., Ye, S., Mason, R.J., parsons, P.E. 1999. Serum surfactant protein –A levels predict development of ARDS in at-risk patients. Chest. 116: 90S-91S.

Haagsman, H.P. 1998. Interactions of surfactant protein A with pathogens. Biochem Biophys Acta. 1408: 264-77.

Haczku, A. 2008. Protective role of the lung collectins surfactant protein A and surfactant protein D in airway inflammation. J Allergy Clin Immunol. 122: 861-879.

Haeringen, N.Jv. 1993. Inhibition of lysozyme activity by preservatives. Contactologia. 15(E): 98-71

Håkansson K, Lim NK, Hoppe HJ, Reid KB. 1999. Crystal structure of the trimeric alpha-helical coiled-coil and the three lectin domains of human lung surfactant protein D. Structure. 7(3):255-64.

He, Y., Crouch, E.C., Rust, K., Spaite, E., Brody, S.L. 2000. Porximal promoter of the surfactant protein D gene: regulatory roles of AP-1, forkhead box and GT box binding proteins. J Biol Chem. 275: 31051-60.

Hecht, O., Van Nuland, N.A., Schleinkofer, K., Dingley, A.J., Bruhn, H., Leippe, M., Grotzinger, J. 2004. Solution structure of the pore-forming protein of Entamoeba histolytica. J Biol Cehm. 279: 17834-41.

Holly, F.J. 1973. Formation and rupture of the tear film. Exp. Eye Res. 15: 515-25.

Holly, F.J. 1978. Surface chemical evaluation of artificial tears and their ingredients. II. Interaction with a superficial lipid layer. Contact Intraocular Lens Med J. 4(3): 52-65.

Johnston, S.L., Papi, A., Bates, P.J., Mastronarde, J.G., Monick, M.M., Hunninghake, GW. 1998. Low grade rhinovirus infection induces a prolonged release of IL-8 in pulmonary epithelium. J Immunol.160:6172–6181.

Katyal, S.I., Singh, G., Locker, J. 1992. Characterization of a second human pulmonary surfactant associated protein SP-A gene. Am J Respir Cell Mol Biol. 6: 446-452.

Kawasaki, H., Taira, K., Morris, K.V. 2005. siRNA induced transcriptional gene silencing in mammalian cells. Cell cycle. 4 (3): 442-8.

Kingma, P.S., Whitsett, J.A. 2006. In defense of the lung: surfactant protein A and surfactant protein D. Curr Opin Pharmacol. 6:277-83.

Kolozsvari, L., Nogradi, A., Hopp, B., Bor, Z. 2002. UV absorbance of the human cornea in the 240 to 400 nm range. Invest Ophthalmol Vis Sci. 43: 2165-2168.

Korfhagen, T.R., Sheftelyevich, V., Burhans, M.S., Bruno, M.D., Ross, G.F., Wert, S,.E.. Stahlman, M.T., Jobe, A.H., Ikegami, M., Whitsett, J.A., Fisher, J.H. 1998. Surfactant protein-D regulates surfactant phospholipid homeostasis in vivo. J Biol Chem. 273 (43): 28438-43.

Krol, S., Ross, M., Sieber, M., Kunneke, S., Galla, H.J., Janshoff, A. 2000. Formation of three dimensional protein-lipid aggregates in monolayer films induced by surfactant protein B. Biophys. J. 79: 904-18.

Kuhlmann, U.C., Chwieralski, C.E., Reinhold, D., Welte, T., Buhling, F. 2009. Radiation-induced matrix production of lung fibroblasts is regulated by interleukin-8. Int J Radiat Biol. 85(2):138-43.

Kumar, A., Zhang, J., Yu, F.X. 2004. Innate immune response of corneal epithelial cells to Staphylococcus aureus infection: role of peptidoglycan in stimulating proinflammatory cytokine secretion. Invest Ophthalmol Vis Sci. 45(10): 3513-22.

Liu, C.Y., Zhu, G., Converse, R., Kao, C.W., Nakamura, H., Tseng, S.C., Mui, M.M., Seyer, J., Justice, M.J., Stech, M.E. 1994. Characterization and chromosomal localization of the cornea-specific murine keratin gene Krt1.12. J Biol Chem. 269: 24627-24636.

Lokitz, M.L., Zhang, W., Bashir, M., Sullivan, K.E., Ang, G., Kwon, E.J., Lin, J.H., Werth, V.P. 2005. Ultraviolet-B recruits mannose-binding lectin into skin from non-cutaneous sources. J Invest Dermatol. 125: 166-173.

Lu, L., Reinach, P.S., Kao, W.W. 2001. Corneal epithelial wound healing. Exp Biol Med. 226 (7): 653-64.

McCormack, F.X., Gibbons, R., Ward, S.R., Kuzmenko, A., Wu, H., Deepe, G.S. Jr. 2003. Macrophage-independent fungicidal action of the pulmonary collectins. J Biol Chem. 278: 36250-6

Meloni, F., Alberti, A., Bulgheroni, A., Lupi, A., Paschetto, E., Bianco, A.M., Rodi,G., Fietta, A., Luisetti, M., Baritussio, A. 2002. Surfactant apoprotein A modulates interleukin-8 and monocyte chemotactic peptide-1 production. Eur Respir J. 19: 1128-35.

Merritt, T.A., Amirkhanian, J.D., Helbock, H., Halliwell, B., Cross, C.E. 1993. Reduction of the surface-tension-lowering ability of surfactant after exposure to hypochlorous acid. Biochem. J. 295: 19-22.

Meusel, T.R., Imani, F. 2003. Viral induction of inflammatory cytokines in human epithelial cells follows a p38 mitogen-activated protein kinase-dependent but NF-kappa B-independent pathway. J Immunol. 171:3768–3774.

Mo, Y.E., Kankavi, O., Masci, P.P., Mellick G.D., Whitehouse, M.W., Boyle, G.M., Parsons, P.G., Roberts M.S., Cross, S.E. 2007. Surfactant protein expression in human skin: evidence and implications. Journal of Investigative Dermatology. 127:381-386

Monick, M.M., Yarovinsky, T.O., Powers, L.S., et al. 2003. Respiratory syncytial virus up-regulates TLR4 and sensitizes airway epithelial cells to endotoxin. J Biol Chem. 278:53035–53044.

Mulugeta, S., Beers, M.F. 2006. Surfactant protein C: its unique properties and emerging immunodulary role in the lung. Microbes Infect. 8: 2317-23.

Nadeau, W.J., Pistole, T.G., McCormick, B.A. 2002. Polymorphonuclear leukocyte migration across model intestinal epithelia enhances Salmonella typhimurium killing via the epithelial derived cytokine, IL-6. Microbes Infect.4:1379–1387.

Nakamura, Y., Kano, R., Hasegawa, A., Watanabe, S. 2002. Interleukin-8 and tumor necrosis factor alpha production in human epidermal keratinocytes induced by Trichophyton mentagrophytes. Clin Diagn Lab Immunol. 9(4): 935-937.

Ni, M., Evans, D.J., Hawgood,S., Anders, M., Sack, R.A., Fleiszig, S.M.J. 2005. Surfactant protein D is present in human tear fluid and the cornea

and inhibits epithelial cell invasion by Pseudomonas aeruginosa. Infect Immun. 74 (4): 2147-2156.

Ni, M., Tam, C., Verma, A., Ramphal, R., Hawgood, S., Evans, D.J., Fleiszig, S.M.J. 2008. Expression of surfactant protein D in human corneal epithelial cells is upregulated by Pseudomonas aeruginosa. FEMS Immunol Med Microbiol. 54: 177-184.

Offord, E.A., Sharif, N.A., Mace, K., Tromvoukis, Y., Spillare, E.A., Avanti, O., Howe, W.E., Pfeifer, A.M. 1999. Immortalized human corneal epithelial cells for ocular toxicity and inflammation studies. Invest. Ophthalmol. Vis. Sci. 40:1091-1101.

Palaniyer, N., Ikegami, M., Korfhagen, T. 2001. Domains of surfactant protein A that affect protein oligomerization, lipid structure and surface tension. Comp. Biochem. Phys. A 129: 109-27.

Pérez-Gil J, Keough KM. 1998. Interfacial properties of surfactant proteins. Biochim Biophys Acta. 1408(2-3):203-17.

Phizackerley, P.J., Town, M.H., Newman, G.E. 1979. Hydrophobic proteins of lamellated osmiophilic bodies isolated from pig lung. Biochem J. 183: 731-736

Pivarcsi, A., Nagy, I., Kemeny,L. 2005. Innate immunity in the skin: how keratinocytes fight against pathogens. Curr Immuno Rev. 1:29-42.

Postle, A.D., Mander, A., Reid, K.B., Wang, J.Y., Wright, S.M., Moustaki, M., Warner, J.O. 1999. Deficient hydrophilic lung surfactant proteins A and D with normal surfactant phospholipid molecular species in cystic fibrosis. Am J Respir Cell Mol Biol. 20:90-8.

Poulain, F.R., Allen, L., Williams, M.C., Hamilton, R.L., Hawgood, S. 1992. Effects of surfactant apolipoproteins on liposome structure- implications for tubular myelin formation. Am J Physiol. 262: L730-9.

Pryhuber, G.S., O'Reilly, M.A., Clark, J.C., Hull, W.M., Fink, I., Whitsett,J.A. 1990. Phorbol ester inhibits surfactant protein SP-A and SP-B expression. J. Biol. Chem. 265 (34): 20822-8.

Ramselaar, J.A.M., Boot, J.P., Haeringen, N.Jv., Best, J.Av., Oosterhuis, J.A. 1988. Corneal permeability after instillation of ophthalmic solutions containing local anaesthetics and preservatives. Curr Eye Res. 7(9): 947-50.

Ren, H., Wilson, G. 1994. The effect of ultraviolet-B irradiation on the cell shedding rate of the corneal epithelium. Acta. Ophthalmologica. 72: 447-52.

Restrepo CI, Dong Q, Savov J, Mariencheck WI, Wright JR. 1999. Surfactant protein D stimulates phagocytosis of Pseudomonas aeruginosa by alveolar macrophages. Am J Respir Cell Mol Biol. 21(5):576-85.

Restrepo, C.I., Dong, Q., Savov, J., Mariencheck, W.I., Wright, J.R. 1999. Surfactant protein D stimulates phagocytosis of pseudomonas aeruginosa by alveolar macrophages. Am J Respir Cell Mol Biol. 21: 576-585.

Ringvold, V. 1998. Corneal epithelium and UV-protection of the eye. Acta Ophthalmol Scand. 76: 149-53.

Ryan, M.A., Akinbi, H.T., Serrano, A.G., Perez-Gil, J., Wu, H., McCormack, F.X., Weaver, T.E. 2006. Antimicrobial activity of native and synthetic surfactant protein B peptides. J Immunol. 176:416-25

Ryan, M.A., Qi, X.Y., Serrano, A.G., Ikegami, M., Perez-Gil, J., Johansson, J., Weaver, T.E. 2005. Mapping and analysis of the lytic and fusogenic domains of surfactant protein B. Biochemistry. 44: 861-72.

Sano, H., Chiba, H., Iwaki, D., Sohma, H., Voelker, D.R., Kuroki, Y. 2000. Surfactant proteins A and D bind CD14 by different mechanisms. J Biol Chem. 275: 22442-61

Sano, H., Sohma, H., Muta, T., Nomura, S., Voelker, D.R., Kuroki, Y. 1999. Pulmonary surfactant protein A modulates the cellular responses to smooth and rough lipopolysaccharides by interactions with CD14. J Immunol. 163: 387-95.

Sato, H., Sano, H., Iwaki, D. 2003. Direct binding of toll-like receptor 2 to zymosan, and zymosan-induced NF-ΚΒ activation and TNF-α secretion are down-regulated by lung collectin surfactant protein A. J Immunol. 171: 417-25.

Savkovic, S.D., Koutsouris, A., Hecht, G. 1997. Activation of NF-kappaB in intestinal epithelial cells by enteropathogenic Escherichia coli. Am J Physiol. 273:C1160–C1167.

Shimmura, S., Tadano, K., Tsubota, K. 2004. UV dose-dependent caspase activation in a corneal epithelial cell line. Curr Eye Res. 28 (2): 85-92.

Sloop, G.D., Moreau, J.M., Conerly, L.L., Dajcs, J.J., O'Callaghan, R.J.1999. Acute inflammation of the eyelid and cornea in Staphylococcus keratitis in the rabbit. Invest Ophthalmol Vis Sci. 40(2):385-91.

Snyder, G.D., Oberley-Deegan, R.E., Goss, K.L., Romig-Martin, S.A., Stoll, L..L, Snyder, J.M., Weintraub, N.L. 2008. Surfactant protein D is expressed and modulates inflammatory responses in human coronary artery smooth muscle cells. Am J Physiol Heart Circ Physiol. 294(5):H2053-9

Sugahara, K., Rubin, J.S., Mason, R.J., Aronsen, E.L., Shannon, J.M. 1995. Keratinocyte growth factor increases mRNAs for SP-A and SP-B in adult rat alveolar type II cells in culture. Am J Physiol. 269: L344-L350.

Sun, Y., Hise, A.G.,Kalsow, C.M., Pearlman, E. 2006. Staphylococcus aureus induced corneal inflammation is dependant on toll-like receptor 2 and myeloid differentiation factor 88. Infect Immun. 74(9): 5325-5332.

Takahashi, H., Sano, H., Chiba, H., Kuroki, Y. 2006. Pulmonary surfactant proteins A and D: innate immune functions and biomarkers for lung diseases. Curr Pharm Design. 12: 589-598.

Tenner, A.J. 1999. Membrane receptors for soluble defense collagens. Curr Opin Immunol. 11: 34-41.

Tiffany, J.M. 1991. The viscosity of human tears. Int Ophthalmology. 15: 371-6.

Tiffany, J.M., Pandit, J.C., Bron, A.J. 1996. Soluble mucins and the physical properties of tears. Invest Ophthalmol Vis Sci. 37: S845.

Tiffany, J.M., Winter, N., Bliss, G. 1989. Tear film stability and tear surface tension. Curr. Eye Res. 8: 507-15.

Vaandrager AB, van Golde LM. 2000. Lung surfactant proteins A and D in innate immune defense. Biol Neonate. 77 Suppl 1:9-13.

Venza, I., Cucinotta, M., Caristi, S., Mancuso, G., Teti, D. 2007. Transcriptional regulation of IL-8 by Staphylococcus aureus in human conjunctival cells involves activation of AP-1. Invest Ophthalmol Vis Sci. 48: 270-276.

Virtanen, I., Tervo, K., Korhonen, M., paallysaho, T., Tervo, T. 1992. Integrins as receptors for extracellular matrix proteins in human cornea. Acta Ophthalmol. 202 (Suppl): 18-21.

Wang, Q., Dziarski, R., Kirschning, C.J., Muzio, M., Gupta, D. 2001. Micrococci and peptidoglycan activate TLR > MyD88>IRAK>TRAF>NIK>IKK>NF-KB signal transduction pathway that induces transcription of interleukin-8. Infect Immun. 2270-2276.

Wick, M.J., Madara, J.L., Fields, B.N., Normark, S.J. 1991. Molecular cross talk between epithelial cells and pathogenic microorganisms. Cell. 67:651–659.

Wispe, J.R., Clark, J.C., Warner, B.B., Fajardo, D., Hull, W.E., Holtzman, R.B., Whitsett, J.A. 1990. Tumor necrosis factor-alpha inhibits expression of pulmonary surfactant protein. J Clin Invest. 86:1954-60

Woodworth BA, Neal JG, Newton D, Joseph K, Kaplan AP, Baatz JE, Schlosser RJ. 2007. Surfactant protein A and D in human sinus mucosa: a preliminary report.ORL J Otorhinolaryngol Relat Spec. 69(1):57-60.

Wright, J.R. 2005. Immunoregulatory functions of surfactant proteins. Nat Rev Immunol. 5(1): 58-68.

Wu, H., Kuzmenko, A., Wan,S., Schaffer, L., Weiss, A., Fisher, J.H. 2003. Surfactant proteins A and D inhibit the growth of Gram-negative bacteria by increasing membrane permeability. J Clin Invest. 111: 1589-602.