Surfactant protein A and surfactant protein D in health and disease

ROBERT J. MASON, KELLY GREENE, AND DENNIS R. VOELKER
1Department of Medicine, National Jewish Medical and Research Center, Denver 80206; and Departments of 3Biochemistry and 2Medicine, University of Colorado Health Sciences Center, Denver, Colorado 80262

Mason, Robert J., Kelly Greene, and Dennis R. Voelker. Surfactant protein A and surfactant protein D in health and disease. Am. J. Physiol. 275 (Lung Cell. Mol. Physiol. 19): L1–L13, 1998.—Surfactant protein (SP) A and SP-D are collagenous glycoproteins with multiple functions in the lung. Both of these proteins are calcium-dependent lectins and are structurally similar to mannose-binding protein and bovine conglutinin. Both form polyvalent multimeric structures for interactions with pathogens, cells, or other molecules. SP-A is an integral part of the surfactant system, binds phospholipids avidly, and is found in lamellar bodies and tubular myelin. Initially, most research interest focused on its role in surfactant homeostasis. Recently, more attention has been placed on the role of SP-A as a host defense molecule and its interactions with pathogens and phagocytic cells. SP-D is much less involved with the surfactant system. SP-D appears to be primarily a host defense molecule that binds surfactant phospholipids poorly and is not found in lamellar inclusion bodies or tubular myelin. Both SP-A and SP-D bind a wide spectrum of pathogens including viruses, bacteria, fungi, and pneumocystis. In addition, both molecules have been measured in the systemic circulation by immunologic methods and may be useful biomarkers of disease. The current challenges are characterization of the three-dimensional crystal structure of SP-A and SP-D, molecular cloning of their receptors, and determination of their precise physiological functions in vivo.

host defense; lung injury; alveolar type II cells; collectins

GENERAL FEATURES OF SP-A AND SP-D

Both SP-A and SP-D are calcium-dependent lectins and are important components of the antibody-independent pulmonary host defense system or innate immunity (19, 44, 78, 108). The genes for both of these proteins are found near the locus of the structurally related mannose-binding protein (MBP) on human chromosome 10, which suggests that these proteins are evolutionarily related and serve similar functions. Humans have two genes for SP-A, which code for proteins with minor amino acid alterations in the collagen domain, and one gene for SP-D (33, 102). These two lectins are structurally related and have many properties in common. Both SP-A and SP-D are collagenous glycoproteins and have complex, but different, highly ordered tertiary structures. Their general structure is shown in Fig. 1 (44). The structure for SP-A is very similar to that for serum MBP-A and the complement component C1q (11). These molecules form a bouquet...
Fig. 1. Structural organization of surfactant protein (SP) A and SP-D. Monomeric units of SP-A and SP-D can be conceptually divided into an amino-terminal position, a collagen-like region, a neck, and a carbohydrate recognition domain. Monomers form trimers that, in turn, form higher ordered oligomers of 18 units for SP-A and 12 units for SP-D. Molecules are not drawn to scale. SP-D is significantly larger than SP-A (108). [Modified from Kuroki and Voelker (44) with permission.]

structure composed of 18 monomers that are organized as 6 trimeric units. The structure of SP-D is similar to that of bovine conglutinin, which assembles four trimers to form a dodecamer of identical monomers (7). This produces a symmetric cruciform-shaped molecule with a distance of ~90 nm between the terminal carbohydrate recognition domains (CRDs). The COOH-terminal CRD is highly conserved and is very similar for SP-A, SP-D, MBP, bovine conglutinin and the bovine serum lectin CL43. This CRD unit is primarily, but not solely, responsible for the host defense properties of SP-A and SP-D.

In addition to binding carbohydrate ligands, SP-A has other important and specific properties. SP-A binds surfactant phospholipids, including dipalmitoyl phosphatidylcholine, avidly and participates in the formation of tubular myelin (44, 96). Over 99% of SP-A in lavage fluid is bound to phospholipid. The current estimate for the concentration of SP-A in rat alveolar fluid is 360 µg/ml of total SP-A and 4–11 µg/ml of free SP-A (93). In normal human volunteers, the concentration of total SP-A in alveolar fluid is calculated to be 180 µg/ml (94). SP-A facilitates the adsorption of surface-active material to the air-liquid interface and prevents the inactivation of surface-active material by serum proteins (5, 87). This function is thought to be very important in acute lung injury and is a consideration for future formulations of surfactant for replacement therapy. In addition, SP-A increases the uptake of phospholipids into type II cells for recycling of surfactant and inhibits the secretion of surface-active material by alveolar type II cells (8, 40, 83, 109, 110). Because of these effects on the secretion, adsorption, and uptake of phospholipid, SP-A is thought to be an important regulatory molecule for surfactant homeostasis in the alveolar compartment. SP-A has also been reported to inhibit a lung calcium-independent phospholipase A2 and may decrease the degradation of internalized phospholipids during recycling (14). As might be expected in light of its function in surfactant homeostasis, SP-A is found in the lamellar inclusions in type II cells and in the tubular myelin lattice. However, SP-A is also found in secretory granules of nonciliated bronchial cells, which was unanticipated originally when the function of SP-A was restricted to surfactant metabolism. The expression of SP-A in small airways is species dependent. In rodents, SP-A is found in alveolar type II cells and nonciliated bronchiolar cells along the conducting airways (Fig. 2). However, in humans, SP-A is found in type II cells and a few cuboidal epithelial cells at the alveolar terminal airway junction but infrequently in the normal respiratory epithelium that lines the conducting airways. In addition, immunocytochemistry also reveals SP-A-reactive material in human tracheal glands (34; Mason, unpublished observations).

SP-D has some properties that differ from SP-A, and, conceptually, this protein should be thought of as a protein distinct from the surfactant system. SP-D does not bind the phospholipids of surface-active material avidly, is mostly soluble in alveolar fluid, and can easily be removed from surfactant by centrifugation. SP-D does bind two lipids with carbohydrate motifs, phosphatidylinositol and glucocereamide, but not phosphatidylcholine (67, 75). The functional importance of this restricted lipid binding of SP-D has not been defined physiologically, and the binding can be inhibited with carbohydrate competition. The location of SP-D in the lung also suggests that it is not directly involved with the surfactant system. SP-D is found in the endoplasmic reticulum of type II cells and in the secretory granules of Clara or nonciliated bronchiolar cells but not in the lamellar bodies of type II cells or in tubular myelin (6, 101). Like SP-A, SP-D is highly expressed in conducting airways of rodents but sparsely in the conducting airways in humans (Fig. 2). In contrast to SP-A, SP-D is not found in human tracheal glands (Mason, unpublished observations).

The precise functions of these two proteins will be better defined once knockout mice with null alleles for these two genes are studied in detail. The SP-A knockout mouse (SP-A°) is apparently normal in a clean environment, and there is relatively little alteration in surfactant homeostasis (26, 35). However, there are alterations in host defense capabilities (45). These observations suggest that a major function of SP-A is related to host defense. The role of SP-A in surfactant
homeostasis is unclear. The near-normal function of the biophysical component of the surfactant system in animals with null alleles indicates that SP-A is not essential to the process. One interpretation of these findings is that the in vitro measurements of SP-A function in the surfactant system do not reflect the in vivo properties. Alternatively, the SP-A functions observed in vitro may also occur in vivo and be duplicated by other proteins. If the latter is true, it would be consistent with both the in vitro and in vivo observations. SP-A may play an important role in surfactant function when the system is stressed, as in acute lung injury. In support of this concept, surfactant isolated from the SP-A-deficient mouse is more susceptible to inactivation by serum proteins than surfactant from wild-type mice, although the in vivo properties were similar (27). The phenotype of the SP-D knockout mouse (SP-D°) has not yet been reported. However, because both proteins have similar host defense properties, the double knockout strains (SP-A° and SP-D°) may be required to demonstrate the critical importance of these proteins in innate immunity. We believe that the difference in distribution of SP-A and SP-D between humans and rodents will be important in the pathogenesis of airway diseases in humans and mice. In humans, SP-A and SP-D are restricted to the gas-exchange units of the lung and are found only sparsely in the respiratory epithelium of the conducting airways, whereas in rodents, both SP-A and SP-D are found in the conducting airways. To date, no human diseases have been attributed to deficiencies in these two proteins. One would hypothesize that the diseases should present with susceptibility to local infection in the lungs but not to systemic infections as occurs with a deficiency of MBP, a major circulating protein of innate immunity.

Fig. 2. Immunocytochemistry of SP-A (A, C, E, and G) and SP-D (B, D, F, and H). Purpose of micrographs is to demonstrate that SP-A and SP-D are much more highly expressed in airway cells in rats and mice than in humans and that SP-A and SP-D are highly expressed in hyperplastic type II cells in interstitial lung disease. A and B: SP-A and SP-D, respectively, from normal human lung fixed with acid alcohol. SP-A and SP-D were detected with monoclonal antibodies with a standard horseradish peroxidase technique and brown diaminobenzidine reaction product. Arrow, rare epithelial cells in terminal airways that stain for SP-D. C and D: SP-A and SP-D, respectively, in adjacent sections of a biopsy from a patient with pulmonary fibrosis. Hyperplastic type II cells contain both SP-A and SP-D, and distribution of these proteins in different cells is similar. E and F: SP-A and SP-D, respectively, from normal rat lung. These sections are fixed with 4% paraformaldehyde, and SP-A and SP-D were detected with a microwave antigen-retrieval system and polyclonal rabbit IgG to the respective antigen. G and H: SP-A and SP-D, respectively, from normal mouse lung. These lungs were fixed with acid alcohol and detected with polyclonal antibodies to rat SP-A and SP-D. Dominant staining is in airway cells, although there is weaker staining in alveolar walls, presumably type II cells. Arrows, alveolar epithelial cells that stain positively for SP-A (E and G) and SP-D (F and H). Magnification in all micrographs is identical. Bar, 50 µm.
STRUCTURE AND FUNCTION

The overall structure of SP-A and SP-D is similar, and the primary structure consists of four domains (see Fig. 1). The amino-terminal segment contains cysteines that form interchain disulfide bonds that cross-link subunits to form covalent oligomeric structures. Moving from the NH2 to the COOH terminus, the next adjacent domain is the collagen-like region that promotes the formation of noncovalent trimers among monomeric subunits. The collagen region also imparts a rigid longitudinal structure to the molecules and organizes the spatial distribution of the carboxy-terminal CRDs. The collagen-like domain of SP-D is linear, but that of SP-A has a kink halfway through this region. Adjacent to the collagen domain is the neck region that forms a coiled coil motif and plays an important role in the folding and organization of the CRD (104). The COOH-terminal domain is the CRD that contains calcium- and carbohydrate-binding sites.

SP-A

The structural domains of SP-A have been mapped extensively. Most of the structure and function studies have focused on the lipid-binding properties of SP-A and interactions with alveolar type II cells. Chemical modification studies initially identified basic amino acid residues as important determinants in the interaction between SP-A and its receptor on alveolar type II cells (41). McCormack et al. (53) used the baculovirus-SF9 cell system to prepare recombinant proteins to define structural elements necessary for specific functions. The initial study demonstrated the important role of the collagen-like domain in the formation of large oligomers and in the thermal stability of the protein (53). Numerous site-directed mutations have defined the protein domains and specific amino acids necessary for SP-A functions. In SP-A mutants in which the N-linked oligosaccharides were genetically deleted, the interactions with type II cells and phospholipids remain normal (41). These results demonstrate that the carbohydrate moieties of SP-A are not essential for many of its activities in terms of lipid binding and interactions with type II cells. However, these carbohydrate moieties are important for binding to mycobacteria, influenza virus, and herpes simplex virus type 1 (2, 15, 72, 100). Mutations introduced into the NH2 terminus to substitute serine for cysteine at position 6 (C6S) and deletion of the collagen domain (removal of amino acids 8–80 (ΔG8-P80)) alter the ability of SP-A to form oligomers and the interactions with type II cells and lipids (58). The C6S mutation reveals that a subpopulation of molecules contains an additional NH2-terminal cysteine consistent with a recent report (13) of a novel processing variant of mature SP-A. The C6-dependent cross-linking of monomeric SP-A plays an important role in lipid binding and appears essential for lipid aggregation and uptake by type II cells. The C6S mutant weakly competes for the high-affinity type II cell receptor and only partially inhibits surfactant lipid secretion. The effects of the C6S substitution appear largely attributable to changes in protein oligomerization. Although trimerization of the collagen domain is thought to be important in the assembly of mature SP-A oligomers, the ΔG8-P80 mutant (the collagen deletion mutant) remains highly oligomeric, perhaps even forming 18mers (58). The NH2-terminal cysteines and the triple helical neck contribute significantly to the formation and stability of the resultant oligomers. The ΔG8-P80 mutant binds lipid less efficiently than the wild-type SP-A but aggregates lipid equally well. As predicted from an earlier study (53), lipid aggregation effected by this mutant is thermostable. The ΔG8-P80 protein does not bind the high-affinity SP-A receptor on type II cells and does not specifically inhibit surfactant secretion.

Extensive mutational analysis has been applied to the CRD of SP-A. Much of the work is predicated on extrapolation from the crystal structure of MBP-A with its bound ligand (105). McCormack et al. (57) created tandem point mutations, E195Q and R197D, to alter the carbohydrate-binding specificity of the CRD region from mannose to galactose and demonstrated that the carbohydrate-binding specificity is intimately linked to binding alveolar type II cells and regulating surfactant secretion. These tandem mutations to SP-A also eliminate lipid aggregation and uptake into type II cells. The E195Q and R197D mutants will continue to be useful in future studies in which it is necessary to define the relationship between carbohydrate binding and newly described functions as a host defense molecule. Alanine mutagenesis of conserved amino acids in the predicted calcium- and carbohydrate-binding sites (residues E195, E202, N214, and D215) leads to marked reductions in interactions with type II cells and phospholipids (59). Among these mutations, the N214A substitution retains near wild-type activity for lipid binding and aggregation. These results further implicate the CRD as an important element in both lipid and type II cell interactions. Additional point mutants in the CRD at position 197 indicate that a basic amino acid is not required at this position for interactions with type II cells and lipids (73). However, the basicity of residue 197 can dramatically enhance (R197K) or inhibit (R197H) lipid uptake (73).

Kuroki et al. (42) and Ogasawara et al. (68) created chimeras between SP-A and SP-D to define structural determinants within these two collectins, and these chimeras have proven valuable for mapping functional domains within this protein family. Swapping the four major domains (NH2 terminus, collagen, neck, and CRD) of SP-A and SP-D implicates the neck and CRD elements of SP-A in lipid binding and inhibition of secretion in vitro. The SP-A-SP-D chimeras have also facilitated the mapping of monodonal antibody epitopes (42). One monodonal antibody (6E3) that recognizes the neck region of SP-A blocks lipid uptake into type II cells but not lipid binding, aggregation, or inhibition of secretion in vitro. A second monodonal antibody (1D6) recognizes the CRD and blocks SP-A interactions with phospholipids, enhancement of cellular uptake, and inhibition of secretion.
Recently, Honma et al. (25) created SP-A-MBP-A chimeras that yielded surprising results. MBP-A does not interact with lipids or type II cells, and substitution of portions of SP-A with MBP-A elements was hypothesized to eliminate SP-A functions. However, nested chimeras containing MBP-A elements within the CRD of SP-A retain significant SP-A activity. This result was completely unanticipated because the longest SP-A region replaced contains the sequence that has the major binding sites for lipids and type II cells. Nevertheless, the results clearly demonstrate that the active site of MBP-A can functionally replace that of SP-A. The results further indicate a surprising level of plasticity among the C-type lectin family members and suggest that conformational constraints placed on the COOH-terminal portion of MBP-A by SP-A dramatically alter the function of this segment of MBP-A.

SP-D

The mutational analyses of SP-D defined the requirements for oligomer formation and the importance of the CRD domain. The NH2 terminus of SP-D contains two cysteines involved in covalent oligomerization of the molecule and assembly into mature dodecamers (19). Brown-Augsburger et al. (3) made the tandem substitutions, C15,20S, to produce molecules that are incapable of forming covalent cross-links but are assembled as trimers via triple helical formation of the collagen and neck domains. The C15,20S mutant exhibits increased thermal lability and protease susceptibility. This protein binds to influenza A virus but fails to aggregate the virus and enhance its binding to neutrophils. As in the case of SP-A, the full oligomerization of SP-D appears to play an important role in maintaining biological potency that may result from specific spatial cross-link of ligands as well as from amplification of relatively weak interactions via multiple combining sites. Molecular genetic deletion of the collagen domain (ΔL27-P202) yields an SP-D molecule that forms covalent trimers and exhibits nearly wild-type levels of binding to mannose-Sepharose and phosphatidylinositols (70). Binding of the mutant protein to glucosylceramide is reduced by 50%. Treatment of SP-D with collagenase removes both the NH2 terminus and collagen domain, and the resulting molecule fails to bind glucosylceramide and weakly binds mannose-Sepharose and phosphatidylinositol (70). Recombinant molecules truncated to contain only the neck and CRD domains have also been produced (46), and these proteins retain binding to Klebsiella pneumoniae, Pseudomonas aeruginosa, and Escherichia coli that is inhibited by maltose and EDTA. These binding studies indicate that the ligands on bacteria are carbohydrate and further establish the importance of the CRD domain in SP-D function. Mutagenesis of the putative active site involved in both calcium and carbohydrate binding has focused on amino acids E321 and N323. Ogasawara and Voelker (69) introduced the tandem substitutions E321Q and N323D into SP-D for the purpose of altering the carbohydrate specificity of this protein as had previously been done for MBP-A (105) and SP-A (57). This modified SP-D with the double mutation lost the ability to bind mannose-Sepharose but did acquire galactose-Sepharose-binding activity as expected. Further investigation of the E321Q,N323D protein revealed that it retained 50% of its phosphatidylinositol binding but lost glucosylceramide binding. This latter finding indicates that the binding sites for the two lipid ligands may partially overlap but are not identical. Several ligand-binding experiments demonstrated that the mutant protein acquired increased affinity for galactose despite the inability to use galactose-Sepharose as an affinity purification matrix. The results obtained with the E321Q,N323D mutant clearly identify these residues within the CRD as important elements in the recognition of lipid and carbohydrate ligands.

Although the dominant form of human SP-D is composed of monomeric subunits with a reduced molecular mass of 43 kDa, in some individuals there is also a monomeric subunit with an apparent molecular mass of 50 kDa under reduced denatured conditions (52). This 50-kDa variant of the carboxy-terminal collagenase-resistant fragment has the same NH2-terminal sequence, amino acid composition, and apparent size as the 43-kDa subunit. The major difference is apparently due to posttranslational processing and differential glycosylation in the NH2-terminal portion of the molecule. The 50-kDa form does not form higher ordered oligomers and is, therefore, not found in SP-D purified by gel filtration. This variant has not been found in rat SP-D.

COMMENTS

Although mutagenesis of both SP-A and SP-D continues to provide valuable new information, the most important details about their protein structure must ultimately come from solution of their crystal structure. This remains a very high priority for researchers interested in these proteins and their multiple ligands. The diverse nature of the ligands that interact with SP-A and SP-D makes cocryotillation with these ligands important for mapping the exact amino acid contact sites. A clear understanding of these proteins at the atomic level will raise the possibility of deliberate protein engineering strategies to enhance or broaden the spectrum of pathogens recognized by these proteins.

The other important priority is to define the physiological abnormalities in mice with genetically deleted SP-A and SP-D. The observation that surfactant homeostasis was apparently unaltered in the SP-A-deficient mice was unanticipated in light of the documented effects of SP-A in vitro (26, 35). We feel that more detailed studies are required to define potential alterations in surfactant metabolism in these mice. Like many important biological systems, there may be redundancy such that, in the normal state, there are no physiological abnormalities and that deficiencies only become apparent under conditions of stress such as acute lung injury. Recently, Ikegami et al. (27) have demonstrated that the surfactant from SP-A-deficient mice is more sensitive to inhibition by serum proteins.
and has fewer large aggregates. The importance of these proteins will require stressing the pulmonary system as in acute lung injury and as well-defined exposures to infectious organisms.

**BINDING TO INFECTIOUS ORGANISMS**

SP-A and SP-D are important host defense molecules and bind a variety of organisms, usually through their CRD domains (19, 44, 108). Although their carbohydrate-binding properties are similar, they are not identical. Hence these proteins bind with different affinity and possibly to different ligands on microorganisms. In addition, there are several reports of SP-A binding to organisms via its N-linked carbohydrate. In terms of monosaccharide binding, SP-A has a preference for mannose and fucose, whereas SP-D has a preference for maltose, glucose, and mannose (46, 47, 74). There are few reports on the relative affinity of SP-A or SP-D to complex carbohydrates on cell surfaces.

**VIRUSES**

SP-A and SP-D bind influenza and herpes simplex viruses (2, 21, 22, 49, 99, 100). Hartshorn et al. (22) demonstrated that both proteins bind influenza A virus and inhibit hemagglutination. SP-A and SP-D also inhibited the increase in the respiratory burst by neutrophils due to influenza infection. The decrease in respiratory burst is thought to be partially responsible for the development of bacterial infections that follow influenza pneumonia. The influenza virus binding was calcium dependent and inhibited by sugars. Malhotra et al. (49) also found that SP-A binds influenza virus in a calcium-dependent manner but, in addition, observed some SP-A binding that was calcium independent. Neuraminidase appears to be the principal calcium-dependent ligand for SP-A on the virus. Benne et al. (2) also reported that SP-A binds to cells infected with influenza A virus. However, the binding was not inhibited by sugars but was inhibited by removal of the N-linked carbohydrate of SP-A or the terminal sialic acid residues. Human SP-D also agglutinates influenza A virus, inhibits hemagglutinin, enhances neutrophil binding, and inhibits the viral-induced diminution of the neutrophil respiratory burst (22). In addition, human SP-D inhibits viral infectivity in vitro (21). There is the suggestion that the higher ordered oligomers of SP-D, the stellate or astral form, bind more virus than the usual dodecameric form of SP-D that has only four trimers (21). In addition, mutants of SP-D that cannot form the higher ordered oligomers fail to inhibit hemagglutination by influenza A virus (12). These interactions with influenza virus are inhibited by maltose or EDTA, which indicates that the binding site is in the CRD domain. Recently, Reading et al. (82) demonstrated the importance of SP-D in vivo in protection against influenza virus infection in mice.

Van Iwaarden et al. (100) reported that SP-A binds to herpes simplex virus type 1 infected cells and that removal of the N-linked carbohydrate on SP-A inhibits this binding. SP-A increases the phagocytosis of herpes simplex virus type 1 by alveolar macrophages, and this effect is inhibited by an antibody to SP-A but not by C1q (99), which implies that SP-A did not bind via the C1q receptor. Guay et al. (18) found no effect of SP-A on the rate of entry of human immunodeficiency virus-1 into mononuclear phagocytes. Although the interaction of SP-A and SP-D has been evaluated with only a few viruses, it is likely that SP-A and SP-D will bind to many respiratory viruses. In addition, some viruses appear to bind to the carbohydrate portion of SP-A. There are multiple potential interactions of SP-A and SP-D with viruses, and these include physical clearance, infectivity, and response of host cells to produce cytokines and inflammatory mediators after viral infection.

**BACTERIA**

Both SP-A and SP-D bind to gram-positive and gram-negative bacteria and have been considered important molecules in host defense. Van Iwaarden et al. (95) reported that whole surfactant and isolated SP-A stimulated phagocytosis of serum-opsonized Staphylococcus aureus but did not mediate the uptake of unopsonized Staphylococcus. However, Manz-Keinke et al. (50) reported that SP-A could also stimulate phagocytosis of unopsonized S. aureus but that the effect depended on growth conditions of the organisms and thereby their external coat. SP-A enhanced phagocytosis of logarithmically growing bacteria but not bacteria from stationary cultures. The growth conditions of the organisms are very important variables in studies of the interactions of SP-A and SP-D with bacteria and account for some discordant observations in the literature. Tino and Wright (93) reported that SP-A binds to and increases phagocytosis of Streptococcus pneumoniae and group A Streptococcus. Hence, SP-A appears to be an important host defense molecule for gram-positive organisms, but there is less information on the interaction between SP-D and gram-positive organisms.

Both SP-A and SP-D bind to lipopolysaccharide (LPS) and gram-negative bacteria with the rough form of LPS and will aggregate these bacteria (38, 62, 77, 96). However, only SP-A has been reported to increase phagocytosis and bacterial killing (30, 50, 77). SP-A and SP-D bind the smooth variants of E. coli poorly (39, 96). Van Iwaarden et al. (96) found that SP-A bound to the lipid A moiety of LPS and that this binding requires calcium and is not inhibited by mannan or removing the N-linked carbohydrate from SP-A. Kalina et al. (31) also reported that SP-A binds to the lipid A portion of endotoxin. Kuan et al. (39) reported that SP-D bound and agglutinated E. coli in a calcium-dependent manner that was inhibited by sugars. In addition, the ability of lavage fluid to agglutinate E. coli was inhibited by an antibody to SP-D. SP-D in lavage fluid could selectively be absorbed by incubation with E. coli, which demonstrates the high affinity of SP-D binding to this gram-negative organism. The binding was thought to be due to the interaction of the CRD of SP-D with the core oligosaccharides of the LPS moiety of E. coli. There was much less binding to LPS of the smooth varieties of
E. coli, which implies that the terminal O-polysaccharide of LPS can block or mask the interaction of SP-D and the core oligosaccharide. One possible explanation for the different effect on phagocytosis may be that the CRD of SP-D is involved with both the interaction with E. coli and with macrophages, whereas for SP-A the CRD may not be involved in binding E. coli and may be free to interact with a macrophage receptor. Lim et al. (46) described recombinant neck and CRD domains of bovine SP-D and demonstrated that this mutant, without the NH2-terminal and collagen domains, bound to the LPS of E. coli, Klebsiella, and P. aeruginosa similar to the native protein. Isolated SP-A binds to and increases the phagocytosis of Haemophilus influenzae (93). Bronchoalveolar lavage fluid also increases the phagocytosis of H. influenzae, and this effect was inhibited by an antibody to SP-A but not by an antibody to SP-D. Kabha et al. (30) reported that SP-A increased phagocytosis of K. pneumoniae by two different mechanisms. One was by serving as an opsonin and binding to Manα1Man sequences on the capsular polysaccharide and the other by activating macrophages to upregulate their mannose receptors. The possibility that SP-A could activate macrophages is significant, and macrophage activation occurred independent of endotoxin, which is sometimes associated with the isolated SP-A. SP-A and SP-D appear to bind P. aeruginosa poorly (93). Pseudomonas is a major pathogen in cystic fibrosis and nosocomial infections.

**MYCOBACTERIA**

Gaynor et al. (15) investigated the interactions between SP-A and macrophages and Mycobacterium tuberculosis. They found that SP-A in solution enhanced the adherence and subsequent phagocytosis of mycobacteria by macrophages. This effect was attributed to an interaction between the N-linked carbohydrate moieties of SP-A and macrophages and was thought to be due to increased surface expression of the mannose receptor on macrophages. These effects were observed with SP-A isolated from patients with alveolar proteinosis and with recombinant rat SP-A produced in SF9 insect cells but not with native rat SP-A or SP-A from healthy volunteers. Differences in the effects of SP-A are likely due to different sources and methods of preparations and must be kept in mind in interpreting conflicting observations in the literature. However, rat SP-A and SP-A from healthy volunteers did increase adherence of mycobacteria to macrophages in the assay in which SP-A was bound to the glass coverslip onto which the macrophages were plated. In this study, direct binding of SP-A to mycobacteria was not reported nor was the effect of SP-A in the presence of phospholipid investigated. Pasula et al. (72) confirmed the findings of the importance of the carbohydrate portion of SP-A for the binding of SP-A to M. tuberculosis to murine alveolar macrophages. Downing et al. (9) reported that human lavage fluid promoted attachment of M. tuberculosis to murine alveolar macrophages and that this effect was inhibited in part by an antibody to SP-A. Finally, Martin et al. (51) also reported that SP-A increased attachment of Mycobacterium to macrophages and that the increase in SP-A in lavage fluid of human immunodeficiency virus-infected patients accounted for increased attachment of Mycobacterium to macrophages due to whole lavage fluid from these patients. Recently, Weikert et al. (103) reported that SP-A increased the binding and phagocytosis of bacillus Calmette-Guerin with rat macrophages and human monocytes and that this interaction occurred via a 210-kDa SP-A receptor. Studies with mycobacteria and SP-D have not been reported to our knowledge. An important issue is whether SP-A plays an important role in the clearance of pathogenic mycobacteria and whether interactions of SP-A and mycobacteria contribute in part to the virulence of individual isolates.

**FUNGI**

Both SP-A and SP-D may be important in defense against deep fungal infections. Schelzen et al. (86) reported that SP-A and SP-D bind acapsular Cryptococcus neoformans but not encapsulated Cryptococcus. SP-D agglutinated the acapsular yeast and showed much more intensive binding than SP-A (86). This was the first report, to our knowledge, that showed a clear preference for the binding of SP-D over SP-A to an organism. Rosseau et al. (85) reported that SP-A inhibited phagocytosis of serum-opsonized Candida albicans by alveolar macrophages in suspension but not by adherent macrophages. In these studies, there was no indication that SP-A served as an opsonin for Candida. Recently, Madan et al. (48) reported that human SP-A and SP-D bound to Aspergillus fumigatus conidia and enhanced their phagocytosis and killing by human neutrophils and alveolar macrophages. The binding to Aspergillus spores was calcium dependent and inhibited by mannose or maltose.

**PNEUMOCYSTIS**

Both SP-A and SP-D bind to Pneumocystis carinii (15, 66, 111). O’Riordan et al. (66) showed that SP-D binds P. carinii via glycoprotein A (gp120) on the organism surface and that the binding is inhibited by glucose, mannose, or EDTA. Zimmerman et al. (111) also demonstrated that SP-A bound gp120 and that the binding was calcium dependent and inhibited by mannose. SP-A binds the oligosaccharide of the major surface glycoprotein of the organism. Structural mutants of SP-A with alanine mutations in the predicted calcium- and carbohydrate-ligating residues of the CRD (54) fail to bind Pneumocystis. Although SP-A and SP-D bind to P. carinii, they are found in association with the organisms in vivo, and stimulate adherence of Pneumocystis to macrophages, they do not enhance phagocytosis by macrophages (66, 107). In this case, there is a strong association of SP-A and SP-D with the organism but no evidence of enhanced clearance. SP-A levels are elevated in the lavage fluid of patients and rodents with infected P. carinii (76).
COMMENTS

The interactions between SP-A and SP-D with microorganisms and phagocytic cells are complex, and specific considerations are highlighted in Fig. 3. At least two sites on SP-A have been implicated in the interactions with microorganisms. Most binding to microorganisms for both proteins occurs via the CRD unit and is inhibited with sugar or chelation of calcium. However, for influenza and herpes simplex viruses and mycobacteria, binding to the N-linked carbohydrate on SP-A has also been reported. There may be multiple binding sites on individual organisms. Use of genetically engineered recombinant forms of SP-A will be useful to define this binding more specifically. However, attention will have to be paid to the terminal sugar residues on the N-linked carbohydrate in the recombinant proteins. To date, all of the reported binding of SP-D to organisms is via the CRD unit. Binding to both lectins is also dependent on the growth conditions of the organisms. Log-phase bacteria are bound more readily than organisms from static or stationary cultures. How this might affect colonization of bacteria on epithelial surfaces is not known. In some studies, SP-A increased phagocytosis of opsonized but not unopsonized organisms. In vivo, there might be sufficient complement in the respiratory surface fluid to opsonize organisms without the requirement of inflammation and plasma extravasation. Although interactions of SP-A and SP-D binding to gram-negative bacteria and activation of complement have not been reported, MBP can bind to bacteria and activate the alternative complement pathway to enhance serum bactericidal activity (88). Hence there may be interactions of SP-A and SP-D in the presence of complement that might be missed in the usual in vitro serum-free studies. In addition, binding does not always lead to phagocytosis. Although aggregation of organisms may be useful for host defense, one would think that phagocytosis and intracellular killing or activation of complement would be required. Especially for SP-A, it is important to note whether the binding studies have been done in the presence of phospholipid to mimic the conditions in vivo. Although the biochemical specificity of ligands can be defined in the absence of phospholipid, the physiological interpretation needs to be made after the completion of studies done in the presence of phospholipid. Some preparations of SP-A may also activate macrophages directly so that increased phagocytosis may be due to macrophage activation and not directly related to opsonization per se. In addition, there may be multiple binding domains for SP-A and SP-D on microorganisms, and, in vivo, both SP-A and SP-D may be bound to a single organism. Finally, the source of SP-A and its method of isolation are critical to the in vitro observations.

INTERACTIONS WITH PHAGOCYTIC CELLS

Both lectins have been reported to bind to several receptors on macrophages and to stimulate microbicial metabolism as indicated by the production of reactive oxygen species (4, 39, 50, 63, 64, 71, 80, 95, 97, 106). Van Iwaarden et al. (95) found that SP-A stimulates chemiluminescence by rat alveolar macrophages but not by peritoneal macrophages or neutrophils. The binding of SP-A to alveolar macrophages appears to be specific and, in another study (71), was not seen with other macrophages such as Kupffer cells, resident peritoneal macrophages, or peritoneal macrophages activated by Corynebacterium parvum. Weissbach et al. (106) found that SP-A increases oxygen radical production by macrophages only when bound to zymosan or a solid surface and suggested that a multivalent interaction between SP-A and macrophages is required to stimulate an oxidative burst. Katsura et al. (32) used both rat and human delipidated SP-A and found that
preincubation of rat alveolar macrophages with SP-A inhibited the respiratory burst in cells stimulated by phorbol myristate acetate or zymosan (yeast walls). Both isolated SP-A and total rat surfactant stimulated chemiluminescence by alveolar macrophages, and this effect was inhibited by antibodies to SP-A. However, the effect of isolated SP-A on production of oxygen radicals by alveolar macrophages was completely inhibited by surfactant phospholipids (97). Mdnntosh et al. (61) reported that butanol-extracted SP-A inhibited tumor necrosis factor-α production by endotoxin-stimulated macrophages and that this effect was not inhibited by phospholipid. However, Kremlev et al. (37) used SP-A isolated by urea extraction and polyacrylamide gel electrophoresis and found that SP-A activated macrophages. In terms of SP-A, the method of isolation is an important determinant on the effects observed in vitro.

Van Iwaarden et al. (97) reported that rat SP-D enhances oxygen radical production by alveolar macrophages but not by peritoneal macrophages. Surfactant lipids did not alter this effect of SP-D. Kuan et al. (38) showed that rat SP-D binds to rat alveolar macrophages through a single class of receptors that is inhibited by EDTA and maltose, glucose, or mannose. They suggested that the binding site is a glycosylated ligand on the macrophage plasma membrane. However, the receptor was not isolated. Miyamura et al. (63) studied binding of human SP-D to bovine alveolar macrophages in the presence of EDTA and found binding independent of the presence of C1q, which suggests that the C1q receptor is not involved. Recently, Holmskov et al. (23) isolated a 340-kDa SP-D-binding protein that is found on the surface of alveolar macrophages and may be an SP-D receptor.

**COMMENTS**

The interactions between these lectins, organisms, and inflammatory cells are complicated, as outlined in Fig. 3. As stated above, the growth conditions of the organism will alter surface expression of ligands and thereby their interactions with SP-A and SP-D. Both of these proteins can self-aggregate, and some methods of preparation lead to denaturation. Conceptually, denatured, aggregated SP-A or SP-D would form digestible particles that could activate phagocytic cells. In addition, both of these proteins bind endotoxin avidly, and removing endotoxin from these proteins is difficult. The observation by Kuan et al. (39) that gram-negative bacteria could quantitatively remove SP-D from bronchoalveolar lavage fluid suggests that binding might be quite strong. Hence the knowledge of how much endotoxin is present in preparations of SP-A and SP-D is important for the interpretation of studies with these proteins and phagocytic cells in vitro. Similarly, studies with SP-A need to be done in the presence of phospholipids before firm conclusions about its function in vivo can be made. Finally, the interaction of these lectins with phagocytic cells is apparently dependent, in part, on how the lectins are prepared (36, 60). The studies with SP-A and SP-D should be conducted, if possible, with native proteins prepared without the use of denaturing agents such as urea and SDS or delipidation with butanol. Recently, methods that avoid butanol and denaturing agents have been reported (92, 98). It is our understanding that the current discrepancy as to whether SP-A or SP-D is proinflammatory or anti-inflammatory is largely due to different in vitro observations that may be explained by the methods used to purify the proteins. Finally, there are multiple potential receptors on macrophages. These include a novel receptor for SP-A (4), the C1q receptor (64), surface glycoconjugates on macrophages, and organism-specific receptors if the SP-A or SP-D are presented bound to an organism. For example, CD14 could theoretically be a receptor for SP-A or SP-D bound to endotoxin and complexed to endotoxin-binding protein. Conclusive identification and cloning of high-affinity SP-A and SP-D receptor(s) on macrophages remains a high priority to define these interactions more precisely.

**SP-A AND SP-D AS BIOMARKERS IN HUMAN DISEASE**

Because the concentrations of SP-A and SP-D in lavage fluid and serum vary with disease and lung inflammation, these proteins might be useful markers of disease. SP-A or SP-D have been shown to be elevated in the lavage fluid of animals with acute lung injury due to endotoxin instillation or 85% oxygen exposure (60, 65, 91). Shimura et al. (90) measured SP-A in tracheal aspirates and sputum of patients with adult respiratory distress syndrome (ARDS) and cardiogenic pulmonary edema and found it elevated in both conditions. The concentration in sputum and tracheal aspirates was 200- to 300-fold higher than that in serum. Lavage fluid of patients with idiopathic pulmonary fibrosis (IPF) reflects reduced levels of SP-A and little change in SP-D (24, 55, 56). The reduction in SP-A in lavage fluid was found in a variety of diffuse interstitial lung diseases. The ratio of SP-A to phospholipid was used to correct for recovery of total surfactant and was low in patients with IPF. This ratio predicted the subsequent 5-yr survival of patients with IPF better than any other lavage fluid constituent measured (55). However, the SP-A-to-phospholipid ratio was not a better predictor of survival than a clinical-physiological-radiological score or total lung capacity, which remain clinically useful predictors of survival (55). Hamm et al. (20) reported an increase in SP-A levels in the lavage fluid of patients with sarcoidosis or hypersensitivity pneumonitis. In patients with ARDS, SP-A is reduced in lavage fluid, and the reduction in SP-A correlates with the severity of ARDS (17, 79). In addition, Baughman et al. (1) reported a decrease in SP-A in the lavage fluid from patients with bacterial pneumonia.

SP-A and SP-D have also been measured in serum (10, 24, 28, 43) and may serve as biomarkers of lung disease, especially when alveolar epithelial integrity is compromised. Both proteins have been identified in serum by sandwich ELISAs that use monoclonal antibodies, and several groups with different reagents have found similar absolute values of serum concentration (10, 16, 24, 28, 43). Serum concentrations of SP-A and
SP-D are increased in patients with alveolar proteinosis, IPF, and ARDS (10, 16, 24, 43). Greene et al. (16) have measured SP-A and SP-D in the serum of patients with interstitial lung disease. SP-D levels were elevated in patients with IPF (391 ng/ml; n = 143) and ARDS (61 ng/ml; n = 21) compared with normal subjects (39 ng/ml; n = 45). SP-A levels were also increased in patients with ARDS (61 ng/ml; n = 21) and IPF (92 ng/ml; n = 143) compared with normal subjects (39 ng/ml; n = 46). The mean levels of SP-A and SP-D in patients with sarcoidosis did not differ from those in normal subjects. Importantly, serum SP-D levels predict long-term survival in patients with IPF. There is also a suggestion that changes in the serum value of SP-D might predict the clinical course and response to therapy in IPF (24). The reason for the increase in SP-A and SP-D in serum in these diseases is not known but is probably a combination of type II cell hyperplasia with a concomitant increase in the synthesis of both proteins and spillover into the circulation due to patchy loss of the epithelial barrier. Because the structurally related proteins MBP and conglutinin are serum proteins, it is plausible that SP-A and SP-D might not be rapidly cleared once they enter the circulation. Measuring SP-A and SP-D in serum might be useful in the diagnosis of alveolar proteinosis because the serum levels are quite high, the image on computed tomography scanning is nearly specific, and a biopsy could be avoided. More clinical correlations are needed between serum values of SP-A and SP-D and the usual clinical variables to judge their value in the care of patients with interstitial lung disease and ARDS. The most promising uses would be to predict the development of ARDS in patients at risk for ARDS and identify a subset of patients with pulmonary fibrosis with a poor prognosis for more aggressive therapy. Measurement of SP-A and SP-D in pleural fluid might also be useful to distinguish metastatic adenocarcinoma of the lung from other adenocarcinomas or mesothelioma (89).

CONCLUSIONS AND FINAL COMMENTS

Currently, there remains some controversy about whether native SP-A and SP-D are pro- or anti-inflammatory. The different observations may relate primarily to the method of preparation of the proteins and need to be fully resolved by additional studies. However, the healthy lung is typically not inflamed, and chronic inflammation ultimately damages the lung, suggesting that mechanisms exist to minimize the inflammatory response. Based on these considerations, we believe that SP-A and SP-D will prove to have an anti-inflammatory role. The lungs need a special microenvironment to suppress the inflammatory response. Everyday we inhale 17 kg of air in which many chemicals, antigens, and microbes are suspended. Particles of <2 µm can be deposited in small airways and the gas-exchange units of the lung, and there must be an efficient system for handling this burden of foreign material. The critical issue is to clear the material without generating a destructive inflammatory response or awaiting the delay required for specific T cell-dependent immunity. To accomplish this goal, the lungs need a system to remove materials without recruiting or activating inflammatory cells. We propose that SP-A and SP-D serve this function. However, once an infectious organism starts to proliferate and invade, the lung also needs a prompt response to clear the infections and thereby activate the inflammatory response. In this sense, there may be specific roles for SP-A or SP-D bound to an organism or presented with other costimulating molecules to be proinflammatory. For example, there are in vitro observations that SP-A or SP-D attached to a solid support or bound to organisms activates macrophages, whereas SP-A or SP-D in solution or bound to phospholipids does not. To date, the story is incomplete, and the next few years should provide a great deal more information on the physiologic role of SP-A and SP-D as host defense molecules.

We thank Lynn Cunningham for performing the immunocytchemistry, Leigh Landskroner for medical illustrations, and Kathy Ryan Morgan for secretarial assistance.

This work was supported by National Heart, Lung, and Blood Institute Specialized Center of Research in Pulmonary Fibrosis Grants HL-56556, HL-29891, and HL-45286 and was performed in the Lord and Taylor Laboratory of Lung Biochemistry and the Anna Perahia Adatto Clinical Research Center of the National Jewish Medical and Research Center (Denver, CO). K. Greene is a recipient of National Heart, Lung, and Blood Institute Clinical Investigator Development Award HL-03572.

Address for reprint requests: R. J. Mason, Dept. of Medicine, National Jewish Medical and Research Center, 1400 Jackson St., Denver, CO 80206.

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