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Accumulation of Surfactant Protein D in Human Pulmonary Alveolar Proteinosis

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Surfactant protein D (SP-D) is a collagenous calcium-dependent carbohydrate-binding protein that is structurally related to the serum mannose-binding proteins and pulmonary surfactant protein A. SP-D was initially characterized as a biosynthetic product of freshly isolated rat type II cells and first purified in chemical amounts from bronchoalveolar lavage of rats with silica-induced alveolar lipoproteinosis. The present studies describe the characterization of human SP-D isolated from therapeutic bronchoalveolar lavage of patients with pulmonary alveolar proteinosis. Human proteinosis SP-D was extracted from the 10,000 × g pellet of bronchoalveolar lavage with 100 mmol/L glucose or ethylenediamine tetraacetic acid, and specifically bound to and eluted from maltosyl-agarose. The protein cross-reacted with monospecific antibodies to rat SP-D by enzyme-linked immunosorbent assay and immunoblot and eluted near the position of rat SP-D on reverse-phase high performance liquid chromatography. When chromatographed on 4% agarose (A-15M) in the presence of ethylenediamine tetraacetic acid, the solubilized human proteinosis SP-D eluted near the void volume and earlier than rat SP-D dodecamers or human SP-D multimers in the lavage supernatant. Two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting of proteins in the lavage pellet with antibodies to the carbohydrate-binding domain of proteinosis human SP-D demonstrated covalently cross-linked multimers of SP-D monomers (43 kd, reduced) and multimers of trimeric components stabilized by disulfide and non-disulfide bonds. These studies describe the isolation and biochemical characterization of human SP-D and demonstrate the abnormal accumulation of this protein in the air spaces of patients with alveolar proteinosis. (Am J Pathol 1993, 142:241–248)

Epithelial cell-derived proteins play critical roles in regulating the surface active and permeability properties of the alveolar and bronchiolar epithelium and are probably important in the lung's defense against inhaled microorganisms. In certain pathological states, there is an excessive accumulation of epithelial lining material with large amounts of surfactant-rich lipoproteinaceous exudate within the alveoli and alveolated airways. Human pulmonary alveolar proteinosis is usually idiopathic; however, morphologically similar reactions can be observed in lung injury, particularly in association with massive inhalation of particulate materials including crystalline silica.

Ultrastructurally, the alveolar exudate consists of tubular myelin-like structures, membranous vesicles, amorphous granular material, and a few lamellated bodies.[1–4] Biochemical and immunological analyses of insoluble material from lavage of patients with alveolar proteinosis have demonstrated surfactant phospholipids and proteins, specifically surfactant protein A (SP-A).[5–7]

We have recently identified a distinctive collagenous glycoprotein, designated SP-D.[8–10] SP-D is synthesized by freshly isolated rat type II pneumocytes[8,9,11] and has been immunologically localized to type II cells and nonciliated bronchiolar cells *in vivo*.[12] Our previous studies have shown that SP-D is a calcium-dependent carbohydrate-binding protein, with a domain structure similar to SP-A and the circulating mannose-binding proteins.[10,13] Recent biochemical and ultrastructural studies indicate that SP-D is assembled as a "spider-like" molecule similar to conglutinin, with four triple helical collagenous arms, each with globular terminal carbohydrate binding domains.[14]

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The majority of SP-D can be isolated as a soluble protein from rat, bovine, and human bronchoalveolar lavage (BAL); however, a fraction of SP-D is isolated in association with pulmonary surfactant.[9] We have recently shown that the accumulation of total lavage SP-D is markedly increased in silica-induced lipoproteinosis in rats and that insoluble SP-D is associated with electron-dense granular air space material.[15] The present manuscript describes the isolation and characterization of SP-D from human alveolar proteinosis lavage.

Materials and Methods

Alveolar Proteinosis Lavage

Proteinosis lavage was obtained as a by-product of therapeutic lavage of a middle-age man with a clinical and pathological diagnosis of idiopathic pulmonary alveolar proteinosis. Samples of lavage from an additional proteinosis patient were obtained as a gift from Dr. S. Hawgood (UCSF). Aliquots of unfractionated lavage were stored frozen for subsequent analysis. However, the majority of the lavage was immediately subjected to centrifugation at 10,000 × *g* for 30 minutes at 4 C. The supernatants and pellets were frozen and stored at −20 C. Lavage samples were also obtained from healthy adult volunteers. After centrifugation at 1000 × *g*, supernatants were stored as above.

Purification of SP-D

SP-D in the 10,000 × *g* lavage supernatant or ethylenediamine tetraacetic acid (EDTA) extracts of the 10,000 × *g* pellet were isolated by affinity chromatography on maltosyl-agarose, followed by gel filtration chromatography on 4% agarose in the presence of EDTA.[10] SP-D associated with the 10,000 × *g* pellet was solubilized by repeated extraction with Tris-buffered saline containing 10 mmol/L EDTA before affinity chromatography in the presence of 2 mmol/L calcium. For some studies, SP-D was further purified by reverse phase high performance liquid chromatography (HPLC).[8,9] Briefly, samples were applied to a C4 reverse-phase column (Bio-Rad Richmond, Ca) equilibrated with 30% acetonitrile–0.1% trifluoroacetic acid and eluted with a linear gradient of 30 to 70% acetonitrile in 0.1% trifluoroacetic acid. SP-D was also quantified by reverse-phase HPLC using the integrated peak area at 214 nm and an extinction coefficient derived by amino acid analysis of HPLC-purified rat SP-D (rSP-D).[15]

The bacterial collagenase-resistant domain of SP-D was prepared by incubating HPLC-purified SP-D with highly purified bacterial collagenase using limited modifications of previously described methods.[8] Briefly, ~100 μg of SP-D was heat-denatured for 3 minutes at 100 C and incubated with 2 μg of purified bacterial collagenase for 6 hours at 37 C in Tris-buffered saline containing 25 mmol/L $CaCl_2$. The major collagenase-resistant fragment was resolved from bacterial collagenase and smaller cleavage products by reverse-phase HPLC as above, except with a gradient of 20 to 80% acetonitrile. The major collagenase-resistant fragment eluted as a single peak with a retention time of ~40 minutes. SDS-PAGE and silver staining demonstrated a single band of ~18 kd (reduced) that migrated more rapidly in the absence of sulfhydryl reduction. Definitive identification of the domain was confirmed by amino-terminal microsequencing with comparison to known sequences of rat and bovine SP-D.[12,13]

SDS-PAGE and Immunoblotting

Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 5%/10% discontinuous methylenebis(acrylamide) slab gels and silver-stained.[9] Molecular sizes were estimated relative to reduced internal globular protein standards; myosin (200 kd), phosphorylase b (97 kd), bovine serum albumin (68 kd), ovalbumin (45 kd), carbonic anhydrase (29 kd), and β-lactoglobulin (18 kd)(GIBCO BRL, Gaithersburg, MD). Proteins were quantified by dye binding assay in SDS-PAGE buffer using bovine serum albumin as standard.

For two-dimensional (2-D) SDS-PAGE, unreduced proteins were resolved in the first dimension on a 3%/6% slab gel. Selected lanes were excised, horizontally applied to the 2-D gel (5%/10%), and resolved by electrophoresis in the presence of dithiothreitol.[8] Proteins were visualized by silver staining or by immunoblotting using specific antibodies (see below) and an indirect secondary antibody-horseradish peroxidase conjugate detection system with 4-chloro-1-napthol as substrate.[8] The small amounts of immunoreactive protein in normal lavage were detected using a secondary goat anti-rabbit alkaline phosphatase conjugate (Bio-Rad) and NBT/BCIP (Gibco BRL), or using the secondary antibody-horseradish peroxidase conjugate and an enhanced chemiluminescence detection system (ECL, Amersham International, Arlington Heights, IL) according to the manufacturer's instructions.

Antibody Preparation and Characterization

The antibodies to rSP-D have been previously characterized.[9,11,12,15] Polyclonal antisera to HPLC-purified human SP-D (hSP-D) and to the collagenase-resistant domain of the HPLC-purified protein were prepared in rabbits. Briefly, rabbits were immunized by subcutaneous injection with approximately 10 μg of antigen suspended in complete Freund's adjuvant (Sigma Chemical Co., St. Louis, MO), and boosted at 2- to 4-week intervals with 10 μg of protein in incomplete adjuvant. The antisera were titered by enzyme-linked immunosorbent assay and further characterized by immunoblotting as above.[9] Immunohistochemical and immuno-gold labeling studies were performed essentially as described for the rat.[12,15]

Results

Preliminary Characterization of Proteinosis Lavage

Histological examination of a transbronchial lung biopsy performed before therapeutic lavage demonstrated diffuse intraalveolar granular eosinophilic air space exudates without significant inflammation. Transmission electron microscopy of insoluble material isolated by lavage showed numerous osmiophilic membranous and myelin-like structures, scattered lamellated bodies, and abundant amorphous to granular electron-dense material and small numbers of intact alveolar macrophages. SDS-PAGE and silver staining of the lavage pellet following solubilization in SDS-PAGE buffer demonstrated numerous protein components, including major broad bands (35 to 38 kd, reduced) consistent with SP-A monomers and dimers.[5,6,16]

Isolation of SP-D

Maltosyl-agarose chromatography of the lavage 10,000 × *g* supernatant or EDTA or glucose extracts of the lavage pellet yielded a major disulfide-bonded component of 43 kd (reduced) that comigrated with rSP-D and several minor higher molecular weight components (Figure 1, lane 3). The 43-kd species was specifically degraded by purified bacterial collagenase (Figure 1, lane 4) and cross-reacted with antibodies to rSP-D (see below). Comparable results were obtained for lavage from a second proteinosis patient (data not shown).

Human proteinosis SP-D was separated from contaminating SP-A and lower molecular weight proteins

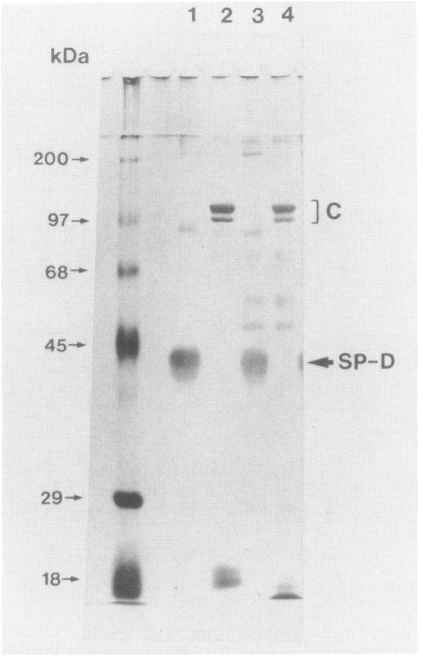

Figure 1. *Purification of hSP-D. SP-D was extracted from the 10,000 × g lavage pellet of proteinosis lavage and partially purified by sequential maltosyl-agarose affinity chromatography and gel filtration chromatography as described in Materials and Methods. Proteins in pooled column fractions were incubated in the presence or absence of purified bacterial collagenase* (C), *reduced with dithiothreitol, resolved by SDS-PAGE, and visualized by silver staining.* Lane 1: *purified human SP-D;* lane 2: *collagenase digest of proteins in lane 1;* lane 3: *lavage proteins eluted from maltosyl-agarose;* lane 4: *collagenase digest of proteins in lane 3. Globular protein standards are at left; the position of migration of reduced rat SP-D (43 kd) is indicated at right. The major collagenous component comigrates with authentic rSP-D; there are also minor higher* M_r, *collagenase-sensitive components with the expected mobility of SP-D dimers and trimers.*

by gel filtration chromatography on 4% agarose using conditions identical to those employed for rSP-D.[9] However, unlike rSP-D molecules (ie, dodecamers of 43 kd subunits) or hSP-D in the lavage supernatant, which eluted within the included volume (Figure 2C), >90% of hSP-D extracted from the proteinosis lavage pellet eluted near the void volume of the column (Figure 2*B*). Reverse-phase HPLC of the void fractions demonstrated a single peak eluting within the gradient near the position of rSP-D (with a retention time of 34.4 minutes versus 32.9 minutes for rSP-D) (data not shown). SDS-PAGE and silver staining of the A15M- and HPLC-purified protein demonstrated a major band comigrating with rSP-D trimers and discrete minor bands that migrated near the top of the separating gel (Figure 3, lane 2). After sulfhydryl reduction, the major band co-migrated with the 43-kd subunit of rSP-D (Figure 3, lane 1); there were also components migrating in the expected positions of covalently cross-linked SP-D dimers and trimers. These components were degraded by purified bacterial collagenase (Figure

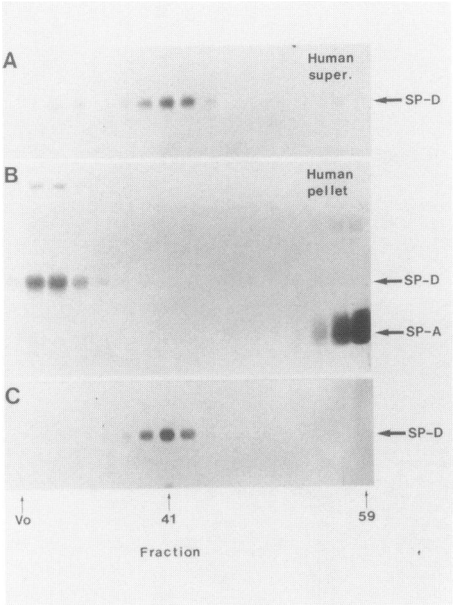

Figure 2. *Gel filtration chromatography of SP-D. SP-D derived from the human lavage supernatant* (**A**), *EDTA extracts of the human lavage pellet* (**B**), *or EDTA extracts of silicotic rat lavage pellet* (**C**) *were separately chromatographed on a column of 4% agarose equilibrated with Tris-buffered saline containing 10 mol/L EDTA as described in Materials and Methods. Aliquots of every other fraction were examined by SDS-PAGE and silver staining. Note that SP-D extracted from the proteinosis lavage pellet elutes near the void volume (V_o) and earlier than rSPD, or hSP-D from the lavage supernatant. Contaminating SP-A is seen in material derived from EDTA extracts of human lavage pellet* (**B**). *Note that lane 4 shows apparent degradation of the major collagenase cleavage product.*

1), and cross-reacted with antibodies to rat and human SP-D (see below).

The pooled proteinosis lavage from our patients contained approximately 12 mg/ml protein, and ~43% of the total protein (5.2 mg/ml) was associated with the 10,000 × *g* pellet. Three successive EDTA extractions of the pellet yielded at least 710 ng SP-D/ml of starting lavage. By contrast, the final recovery of SP-D from the 10,000 × *g* lavage supernatant (as quantified by HPLC following maltosyl-agarose chromatography) was approximately 60 ng/ml of starting lavage. The calculated total recovery of SP-D for approximately 21 l of therapeutic lavage was >16.2 mg. Thus, the majority of SP-D in proteinosis lavage is insoluble under the conditions of lavage and accounted for ~0.014% (by weight) of protein in the lavage pellet.

Structural Comparisons of Rat and Human Proteinosis SP-D

Amino acid analysis of acid hydrolysates demonstrated a similar composition to rSP-D, including the presence of hydroxylysine and 4-hydroxyproline.[13] Bacterial collagenase digestion of purified hSP-D gave an ~18 kd (reduced) peptide similar in size to the collagenase-resistant domain of rSP-D. As for other C-type lectins, the peptide migrated more slowly after reduction, consistent with the presence of intra- but not interchain disulfide bonds (data not shown). Amino-terminal microsequencing of the collagenase-resistant domain demonstrated high homology with the deduced amino acid sequence for the carboxy-terminal globular domain rSP-D and showed nearly complete identity with the corresponding deduced human sequence (Figure 4A).

Peptic digestion was used to further characterize the collagenous domain. Incubation of maltosyl-agarose-purified hSP-D with pepsin for 16 hours at 30 C at pH 2.8 (enzyme/substrate weight ratio of >1:5) gave a stable non-disulfide-bonded fragment that co-migrated with the major pepsin-resistant fragment of rSP-D on SDS-PAGE (~18 kd, collagen standards). Less vigorous digestion conditions yielded slightly larger disulfide-bonded fragments. The major peptic fragment eluted with the same retention time as the pepsin-resistant fragment of rSP-D from reverse-phase HPLC (~38 minutes). Amino-terminal sequencing of this fragment showed

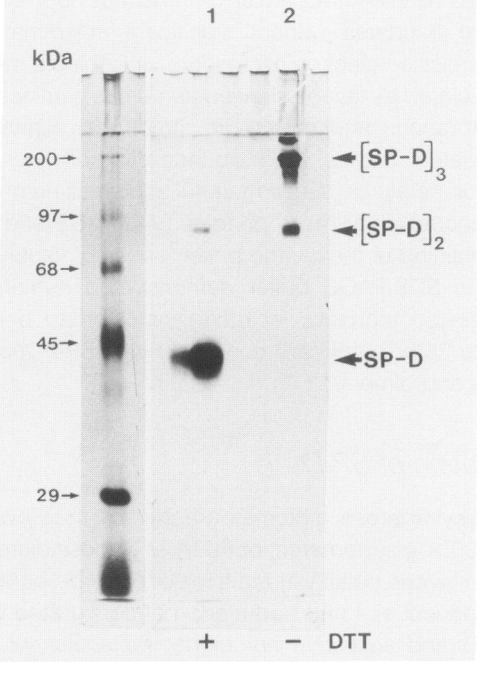

Figure 3. *SDS-PAGE of HPLC-purified SP-D. SP-D was extracted from the lavage pellet and purified by maltosyl-agarose affinity chromatography and gel filtration chromatography on 4% agarose as in Figure 2. SP-D eluting with the void volume of the agarose column was subjected to reverse-phase HPLC as described in Materials and Methods. The protein eluted as a single and apparently homogenous peak with a retention time of approximately 34 minutes. Peak fractions were examined by SDS-PAGE and silver staining with* (**lane 1**) *or without* (**lane 2**) *previous sulfhydryl reduction. Note that unreduced SP-D shows anomalous slow migration on high percentage acrylamide gels consistent with physical retardation by the acrylamide matrix.*[9]

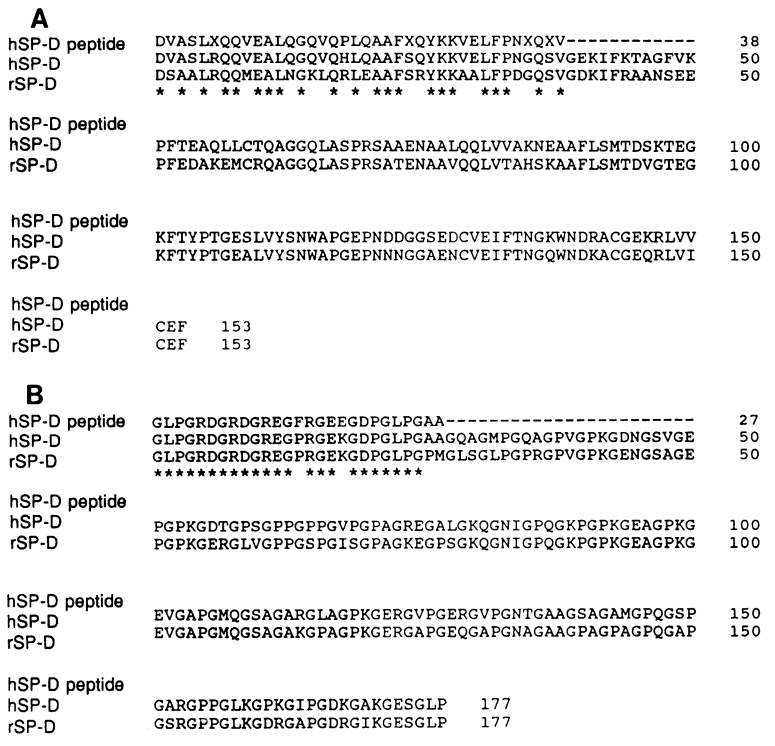

Figure 4. *Sequencing of proteolytic cleavage products of hSP-D. SP-D isolated from the pellet of proteinosis lavage was incubated with bacterial collagenase or porcine pepsin as described in Materials and Methods and Results. The partially purified cleavage products were resolved by SDS-PAGE, electrophoretically transferred to Immobilon membranes, and submitted for gas-phase microsequencing.[1,3] Peptide sequences were compared to deduced amino acid sequences obtained by DNA sequencing of human[1,3] and rat[21] SP-D complementary DNAs.* A: *Noncollagenous sequence from the major collagenase-resistant fragment of SP-D compared with deduced sequence for the carboxy-terminal domain.* B: *Collagenous sequence from the pepsin-resistant fragment of SP-D compared with deduced sequences for the collagen domain. Positions of identity for all three sequences are indicated (*). Note that complementary DNA and genomic sequencing performed subsequent to the publication by Rust et al[1,3] have identified two probable errors in the reported sequence for hSP-D as derived from the H13 complementary DNA.[1,3] The available data predict a Pro rather than a Phe in the X position of the fifth Gly-X-Y triplet of the collagen domain, and the sequence Cys-Glu-Phe (TGCGAGTTC) rather than Cys-Glu-His-Phe (TGCGAGCACTTT) at the carboxy-terminal end of the carbohydrate-binding domain. Additional differences between the complementary DNA and genomic sequences suggest allelic substitutions within the collagen domain.*

only two apparent discrepancies with deduced amino acid sequence for hSP-D (Figure 4B).

Immunological Characterization of hSP-D

Human SP-D cross-reacted with monospecific antibodies to rSP-D by enzyme-linked immunosorbent assay (data not shown) and immunoblot (Figure 5, left). In addition, rat SP-D cross-reacted with polyclonal antibodies to intact HPLC-purified hSP-D (Figure 5, right). For other studies, we immunized rabbits with the monomeric carboxy-terminal domain of hSP-D obtained by bacterial collagenase digestion of HPLC-purified hSP-D (see Materials and Methods). These antisera showed a high titer to antigen and rat and human SP-D by enzyme-linked immunosorbent assay (~1:12,000) and reacted strongly with these components by immunoblot. There was no reaction with purified SP-A, with SP-A in the pellet of proteinosis lavage (see below), or with purified pepsin-resistant collagenous domain of SP-D by immunoblot. On the other hand, enzyme-linked immunosorbent assays demonstrated a titer to native unreduced rat and human SP-A. Preadsorption of the antisera with purified native human SP-A coupled to Affigel-15 (Bio-Rad) decreased the titer to SP-A by a factor of 2. However, adsorption with SP-A was also accompanied by a more than twofold reduction in the titer to SP-D consistent with true immunologic cross-reactivity of conformation-dependent epitopes within the conserved carboxy-terminal domain.

Characterization of SP-D Aggregates

The antisera were used to further examine the state of aggregation of hSP-D in the unfractionated crude surfactant pellet of proteinosis lavage. For this purpose, lavage proteins in the 10,000 × *g* pellet of lavage were solubilized in nonreducing SDS-PAGE buffer, subjected to SDS-PAGE in the absence of dithiothreitol, and resolved by SDS-PAGE in the second dimension after sulfhydryl reduction. The proteins were then visualized by silver staining, or transferred to nitrocellulose before blotting with anti-SP-D (Figure 6).

The profile of silver-stained proteins was comparable with that described in previous studies of proteinosis lavage.[5,6] In particular, the 2-D gels revealed major components corresponding to SP-A monomers and dimers, a disulfide-bonded component that appeared to consist of 52 and 25 kd (reduced) subunits,[5] and albumin (Figure 6, top). The majority of the SP-A migrated near the top of the separating gel in the absence of reduction. Bands corresponding to SP-D were barely visible on the original, heavily loaded, and silver-stained gels. However, immunoreactive SP-D and SP-D multimers (but not

SP-A) were readily identified on the 2-D immunoblots (Figure 6, bottom). The majority of SP-D migrated in the first dimension with the expected mobility of SP-D trimers. However, there were also higher orders of disulfide-bonded aggregates consistent with multimeric complexes of trimers and minor lower M_r components migrating with the expected mobility of disulfide-cross-linked dimers and monomers. Each of the disulfide-bonded multimers showed a subpopulation of chains cross-linked by non-disulfide covalent bonds to yield components that migrated in the second dimension with the expected mobility of SP-D dimers, trimers, and higher aggregates. In addition, the immunoblots showed very minor immunoreactive components that migrated slightly faster than the collagenase-resistant domain of SP-D. There were also minor disulfide-bonded components that migrated slightly faster than SP-D trimers in the first dimension and slightly faster than reduced SP-D dimers and monomers in the second dimension.

The antisera were also used to identify immunoreactive SP-D in normal human lavage. Immunoblotting of 100-µl aliquots of cell-free supernatant of BAL (which contained similar amounts of total protein) from four healthy volunteers demonstrated a single immunoreactive component that comigrated with purified rSP-D in the presence (Figure 7A) and absence of sulfhydryl reduction. Semi-quantitative immunoblot assays using internal SP-D standards suggests that SP-D is present in the 1,000 × *g* supernatant of normal lavage at a concentration of ~50 ng/ml (data not shown). SP-D isolated by maltosyl-agarose affinity chromatography from aliquots of normal BAL comigrated with rSP-D on SDS-PAGE in the absence and presence of sulfhydryl reduction and eluted within the included volume and near the position of rSP-D dodecamers from 4% agarose (A-15M) in the presence of EDTA (data not shown).

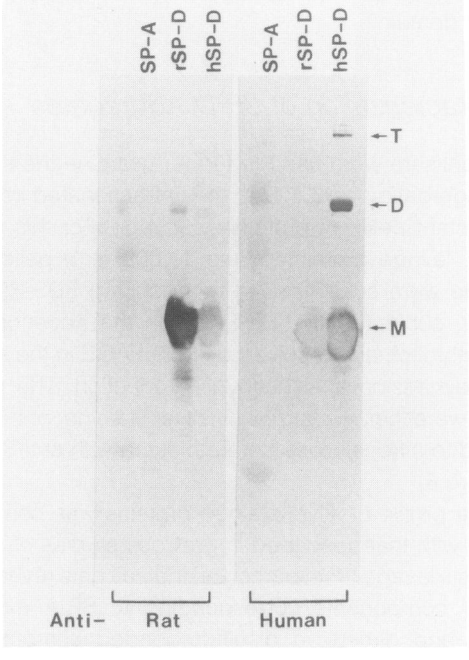

Figure 5. *Immunoblotting of rat and human SP-D. Proteins in EDTA extracts of the 10,000 × g pellet from silicotic rat (rSP-D) or human proteinosis (hSP-D) lavage were resolved by SDS-PAGE, transferred to nitrocellose, and reacted with polyclonal antibodies to HPLC-purified rat* (left) *or human SP-D* (right). *Immunoreactive proteins were detected using an indirect biotinylated secondary antibody/horseradish peroxidase detection system. Note immunoreactive components consistent with SP-D monomers* (M, M_r = 43 kd), *dimers (D), and trimers (T). Prestained globular standards are at the left side of both panels. There was no reaction of either antibody with SP-A.*

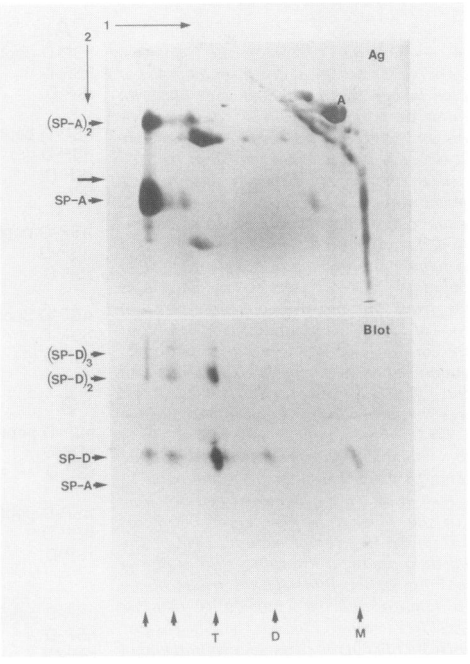

Figure 6. *2-D SDS-PAGE of human SP-D. Aliquots of total proteinosis lavage (~ 500 µg protein) were resolved by 2-D SDS-PAGE in the absence (arrow 1) and presence (arrow 2) of sulfhydryl reduction as described in Materials and Methods. Proteins in one 2-D gel were visualized by silver staining* (top, *Ag*), *proteins in a parallel gel were transferred to nitrocellulose and reacted with antibodies to the collagenase-resistant domain of human SP-D. Immunoreactive proteins were visualized using an indirect biotinylated secondary antibody/streptavidin horseradish peroxidase system using 4-chlorolnapthol as substrate. The positions of migration of* unreduced *monomers (M), dimers (D), and trimers (T) are identified at* bottom. *The positions of migration of* reduced *SP-A monomers and dimers* (top); *and SP-D monomers, dimers, and trimers* (bottom) *are identified at left. The unlabeled arrow at the left of the top panel indicates the expected position of SP-D monomers (43 kd), which were barely visible on the original gel and not discernable in the photograph. The major components on the silver-stained gel correspond to SP-A monomers and dimers and albumin (A).*

Discussion

These studies describe the isolation and biochemical characterization of human SP-D from alveolar proteinosis lavage and the initial characterization of antibodies to intact SP-D and to the carboxy-terminal noncollagenous domain of the human protein. The

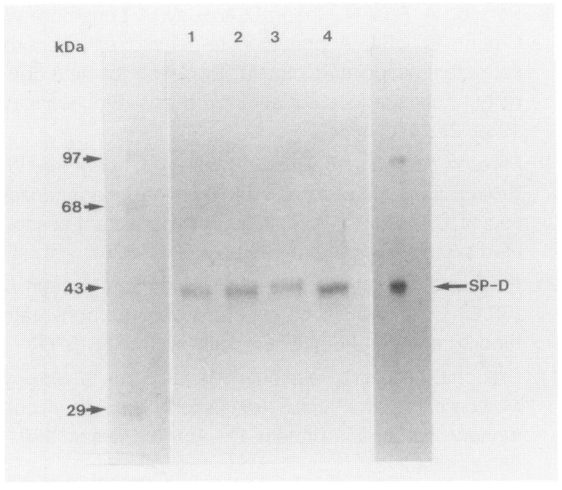

Figure 7. *Immunoblot of normal lavage. Proteins in 100-μl aliquots of the 1000 × g supernatant of BAL from four healthy volunteers* (lanes 1–4), *and an aliquot of proteinosis lavage* (lane 5) *were resolved by SDS-PAGE after sulfhydryl reduction, transferred to nitrocellulose, and incubated with antibody to the collagenase-resistant domain of hSP-D. Immunoreactive proteins were visualized using an indirect biotinylated secondary antibody/streptavidin alkaline phosphatase detection system. Prestained globular protein standards are at* left. *The position of migration of reduced rSP-D is indicated at* right.

latter antibodies were used to characterize crosslinked aggregates of SP-D in proteinosis lavage and to identify immunoreactive SP-D in BAL from healthy adults.

Previous studies of pulmonary alveolar proteinosis have identified collagenous proteins and peptides in association with the insoluble fraction of lavage.[17,18] The major collagenous components correspond to SP-A monomers and dimers.[5,6,16] Thus, the present studies further establish the genetic heterogeneity of collagenous proteins in normal and proteinosis lavage by demonstrating the accumulation of SP-D.

We were unable to accurately quantify the increase in lung SP-D in alveolar proteinosis, in part because of technical differences in the therapeutic and research lavage procedures. However, crude estimates, which correct for the approximate volume fraction of lung lavaged and assume equivalent wash efficiency, suggest that SP-D is increased by at least 50-fold relative to healthy adults. On the other hand, the amount of SP-D relative to other proteins was decreased, since the concentration of total lavage protein (including SP-A and plasma-derived proteins) increased by approximately 100-fold. Although proteinosis lavage provides a convenient source for isolating relatively large amounts of apparently intact human SP-D, we cannot yet exclude that observed differences in aggregation or cross-linking could influence the function of this protein *in vivo* (see below). Furthermore, minor immunoreactive components consistent with SP-D degradation products were identified.

It is not yet known whether the accumulation in human pulmonary alveolar proteinosis reflects increased SP-D synthesis, decreased degradation, or some combination of altered biosynthesis and turnover. Studies of silica-induced lipoproteinosis in rats have demonstrated increased "steady state" levels of total and translatable SP-D messenger RNA within 1 to 3 weeks after silica instillation,[15,19] consistent with increased SP-D gene transcription and protein synthesis. Furthermore, preliminary *in situ* hybridization studies have shown increased signals for SP-D messenger RNA in both type II and Clara cells. As in the animal model, human SP-D was localized primarily to granular electron-dense material without obvious labeling of tubular myelin-like, lamellated, or other membranous structures (data not shown). In any case, the cellular source of the SP-D accumulating in human proteinosis is most likely the type II pneumocyte. Although SP-D and SP-D messenger RNA have been localized to nonciliated bronchiolar cells in rats,[12,20] the vast majority of air space exudate in human proteinosis accumulates distal to the terminal bronchioles.

The mechanism(s) responsible for the highly aggregated state of EDTA-solubilized proteinosis SP-D detected by gel filtration chromatography is uncertain. Rat and human SP-D are highly homologous based on available biochemical and immunological data, and the majority of SP-D associated with the void volume aggregates comigrates with rSP-D (or hSP-D in the lavage supernatant) in the presence and absence of reduction. However, a fraction of total SP-D extracted from the human proteinosis lavage pellet is resolved by 2-D SDS-PAGE as covalently cross-linked complexes of SP-D trimers stabilized by disulfide- and non-disulfide bonds.

The interactions stabilizing the void volume aggregates are not disrupted by EDTA, competing saccharides, extremes of temperature or pH, or nonionic detergents. However, most of the extracted protein elutes within the included volume when chromatography is performed after sulfhydryl reduction and alkylation under non-denaturing conditions. Because the majority of the SP-D comigrates on SDS-PAGE with rSP-D monomers (in the presence of dithiothreitol) or with rSP-D trimers (in the absence of dithiothreitol), we hypothesize that intermolecular interactions among SP-D molecules involve covalent interactions among a small subset of the constituent SP-D chains. Although only a very small fraction of silicotic rat SP-D from the lavage supernatant or pellet elutes with the void volume from A-15M, the formation of EDTA insoluble aggregates has sometimes been observed after prolonged storage of the purified rSP-D (unpublished observations). Higher

orders of disulfide-bonded aggregates could arise by disulfide interchange between SP-D molecules, with the formation of intermolecular sulfhydryl cross-links between the non-collagenous amino- or carboxy-terminal domains, sites of intramolecular interchain disulfide cross-links and intrachain disulfide bonds, respectively.[13,14] Given the presence of significant hydroxylysine in both rat and human SP-D,[9] it is possible that the non-disulfide covalent interactions involve the formation of lysyl-derived cross-links between the collagenous domains. In any case, because virtually all of the SP-D in the proteinosis supernatant elutes near the position of rSP-D from A-15M, we suggest that the formation of SP-D aggregates is related to the accumulation of insoluble air space material. Concentration or ligand-dependent precipitation of SP-D could predispose to cross-link formation as the insoluble air space material accumulates *in vivo*.

Acknowledgments

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