Differences in Biochemical Properties and in Biological Function between Human SP-A1 and SP-A2 Variants, and the Impact of Ozone-Induced Oxidation†

Guirong Wang,‡ Sandra R. Bates-Kenney,§ Jian-Qin Tao,§ David S. Phelps,|| and Joanna Floros*,‡,||,⊥

Departments of Cellular and Molecular Physiology, Pediatrics, and Obstetrics and Gynecology, The Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033, and Institute for Environmental Medicine, University of Pennsylvania Medical Center, Philadelphia, Pennsylvania 19104

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ABSTRACT: The human surfactant protein A (SP-A) locus consists of two functional genes, *SP-A1* and *SP-A2*, with several alleles characterized for each gene. Functional variations between SP-A1 and SP-A2 variants either before or after ozone exposure have been observed. To understand the basis of these differences, we studied SP-A1 and SP-A2 variants by comparing coding sequences, oligomerization patterns under various conditions, composition of oligomers with regard to amino terminal sequence isoforms, biological activity (regulation of phosphatidylcholine (PC) secretion by alveolar type II cells), and the impact of ozone-induced oxidation. We found that (i) the SP-A1 ($6A^4$) allele is the most divergent from all SP-A2 alleles, particularly from the SP-A2 ($1A^1$). (ii) Differences exist in oligomerization among SP-A1, SP-A2, and coexpressed SP-A1/SP-A2, with higher order multimers (i.e., consisting of more subunits) observed for SP-A1 than for SP-A2 variants. Differences among SP-A1 or SP-A2 gene products are minimal. (iii) Amino acid variants in the amino terminal sequences are observed after signal peptide removal, including variants with an extra cysteine. (iv) Oxidation is observed after ozone exposure, involving several SP-A residues that include cysteine, methionine, and tryptophan. (v) The SP-A2 variant ($1A^0$) and the coexpressed protein $1A^0/6A^2$ inhibit ATP-stimulated PC secretion from alveolar type II cells to a greater extent than SP-A1 ($6A^2$), a biologic activity that was susceptible to ozone treatment.

Surfactant protein A (SP-A)[1] has been shown to be involved in surfactant-related functions, exhibiting a variety of roles in innate host defense, the regulation of inflammatory processes in the lung and alterations of the biophysical and biochemical properties of surfactant phospholipid (*1*−*7*). In vitro studies, performed primarily in tissue culture, have shown that SP-A regulates phospholipid turnover by enhancing phospholipid uptake and inhibiting phospholipid secretion. SP-A completely inhibits surfactant secretion regardless of the secretagogue employed (*8*, *9*). The effect is immediate, occurs in the presence of surfactant lipids, and depends on the interaction of the carbohydrate recognition domain of SP-A with the SP-A-specific receptor on the type II cell plasma membrane (*8*−*12*). The role of SP-A in vivo is not as clear because basal phospholipid metabolism in the SP-A gene-deficient mice seems unaffected (*13*, *14*). On the other hand, the absence of SP-A seems to blunt the ability of the lung to respond to stimuli or injury that enhances surfactant uptake in the wild-type animal (*15*, *16*).

Human SP-A consists of two functional genes, *SP-A1* and *SP-A2*, and one pseudogene. More than 30 alleles have been characterized (*17*), and 10 of them appear with greater than 1% frequency in the general population (*17*, *18*). These alleles are classified on the basis of nucleotide differences within the coding region that may or may not result in amino acid changes. Thus, some of the alleles hold the potential for exhibiting functional and/or structural differences among themselves.

SP-A is expressed by epithelial cells, including the type II alveolar epithelial cells in the lung, and is secreted into the lung alveolar space. As the SP-A precursor (*19*, *20*) is processed to mature SP-A, it undergoes several posttranslational modifications including N-linked glycosylation (*19*), hydroxylation of proline residues (*21*), addition of sialic acid to the oligosaccharide, formation of disulfide bonds, and signal peptide cleavage (*1*, *22*). These posttranslational modifications may be important for some SP-A functions (*12*). Isoforms of N-terminal sequence and an alternative start site of translation of rat SP-A have been observed (*23*, *24*).

It has been suggested that native SP-A from bronchoalveolar lavage fluid (BAL) is an octadecamer consisting of

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* Corresponding author: Joanna Floros, Ph.D., Department of Cellular and Molecular Physiology, H166, The Pennsylvania State University College of Medicine, 500 University Drive, Hershey, PA 17033, USA. Telephone: (717) 531−6972. Fax: (717) 531-7667. E-mail: jfloros@psu.edu.

‡ Department of Cellular and Molecular Physiology, The Pennsylvania State University College of Medicine.

|| Department of Pediatrics, The Pennsylvania State University College of Medicine.

⊥ Department of Obstetrics and Gynecology, The Pennsylvania State University College of Medicine.

§ Institute for Environmental Medicine, University of Pennsylvania Medical Center.

[1] Abbreviations: BAL, bronchoalveolar lavage; CHO, Chinese hamster ovary; CRD, carbohydrate recognition domain; GMEM, Glascow's modified Eagle's medium; MSX, methionine sulfoximine; PC, phosphatidylcholine; SP-A, surfactant protein A.

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six trimers, and each trimer is thought to consist of two SP-A1 molecules and one SP-A2 molecule (*25*). SP-A, a C-type lectin or collectin, possesses four regions/domains: an N-terminal region, a collagen-like domain, a neck region, and the carbohydrate recognition domain (CRD). Human SP-A1 and SP-A2 variants collectively differ at 10 amino acid residues, including a cysteine. The different residues are located in the signal peptide region, the collagen-like domain, and the CRD (*18*). SP-A2 has six cysteine residues, but SP-A1 contains an extra cysteine at residue 85 (*20*, *26*). SP-A forms oligomers by means of intermolecular disulfide bonds and/or other molecular interactions and/or noncovalent forces. Two intramolecular disulfide bonds between cysteines 155 and 246, and between cysteines 224 and 238 occur within the CRD domain of SP-A (*27*, *28*). A cysteine at amino acid 26 in rat SP-A is essential for generating oligomeric SP-A (*29*). However, it is unclear whether the extra cysteine at position 85 of SP-A1 plays a role in human SP-A oligomerization by forming intermolecular and/or intertrimeric disulfide bond.

Ozone is a strong oxidizing agent and reacts with unsaturated C=C bonds, amino acids, and other chemical structures. Both biochemical (*30*) and morphological alterations (*31*, *32*) in surfactant have been observed following ozone exposure. Although the order preference for reaction with free amino acids is Cys > Trp = Met > Tyr > His (*33*), differences in the order of amino acid oxidation have been observed in native proteins (*34*) due to their accessibility in the protein secondary structure. Ozone-exposed SP-A exhibits structural and functional changes (*35*−*38*).

Recently, it has been shown that the human single SP-A gene and coexpressed SP-A1/SP-A2 variants are functional and can stimulate TNF-α and IL-8 production by THP-1 cells. Differences in the degree of this stimulation was observed among SP-A variants (*37*, *39*) as well as after ozone-induced oxidation (*37*). The interaction of SP-A variants with type II cells has not been described nor has the molecular/structural basis that may account for differences in biological activity.

In the present study, we characterized in vitro expressed SP-A1, SP-A2, and coexpressed SP-A1/SP-A2 variants, with respect to their molecular oligomerization, isoforms of their amino terminal sequences, oxidation of residues after ozone treatment, as well as the impact of oxidation on properties of SP-A. We used a variety of approaches including amino acid sequence comparison, amino acid microsequencing, and gel electrophoresis under a variety of conditions (reducing, nonreducing, and native). In addition, we compared the biological activity of these SP-A variants with regard to their ability to regulate PC secretion by type II cells, and the effect of ozone-induced oxidation on this process. The SP-A variants used for most of the studies described here were in vitro expressed in the mammalian CHO-K1 cells.

EXPERIMENTAL PROCEDURES

Alignment of SP-A Protein Sequences and Analysis of N-Terminal Signal Cleavage. The alignment of SP-A amino acid sequences were performed using a megAlign program of DNASTAR (version 5.0). The potential sites for N-terminal signal cleavage were analyzed by a PSIGNAL program of PC/gene (version 6.85, IntelliGenetics, Inc.).

Cell Lines, and Cell Culture Conditions. The mammalian Chinese hamster ovary (CHO)-K1 cell line (American Type Culture Collection, Manassas, VA, Cat. No. CCL 61) was used as a host to express human SP-A variants. The cell culture techniques and the culture media were previously described (*37*). In brief, the cells were cultured in Glascow's Modified Eagle's medium (GMEM) (Invitrogen, Carlsbad, CA) plus 10% fetal bovine serum at 37 °C in an atmosphere of 5% CO_2. The stably transfected cell lines from the pEE14-hSP-A transfection were grown in glutamine-free GMEM medium plus 25 μM methionine sulfoximine (MSX). For the double gene stably transfected cell lines from the pEE14-hSP-A and pCI-neo-hSP-A transfection, cells were grown in glutamine-free GMEM plus 300 mg/L of G418 (Invitrogen, Carlsbad, CA). To express SP-A variants, cells were grown to confluence in the growth medium with fetal bovine serum, then growth medium was removed and expression medium, which did not contain fetal bovine serum but has 0.5 mM ascorbic acid and 40 mg of proline/L of medium, was added. The medium containing secreted SP-A protein was harvested after 5 days in culture.

Generation of Stably Transfected Cell Lines. For single gene SP-A variant production, cDNAs of three SP-A1 alleles (6A, $6A^2$, $6A^4$) and three SP-A2 alleles (1A, $1A^0$, $1A^1$) were cloned into the expression vector pEE14 (a kind gift of Lonza Biologics PLC, Slough, UK), and transfected into CHO-K1 cells. For the coexpression of both human SP-A genes, SP-A2 alleles were cloned into the expression vector pEE 14 (*37*, *40*) and SP-A1 alleles were cloned into vector pCI-neo (*41*). Then both constructs were transfected into CHO-K1. After double selection, coexpression cell lines were generated. The stably transfected cell lines expressing SP-A2 variants (1A, $1A^0$, $1A^1$) and coexpressed SP-A1/SP-A2 were described previously (*37*). In the present study, we generated three additional stably transfected cell lines expressing SP-A1 variants (6A, $6A^2$, $6A^4$) using the same techniques and vector (pEE14). Briefly, a 1.3-kb cDNA segment from an SP-A1 allele ($6A^2$, $6A^4$) was inserted into Sma I of the pEE14 vector. This 1.3-kb insert cDNA includes 0.74 kb of coding region, about 0.1 kb 5′ UTR and 0.5 kb 3′ UTR. A 0.9-kb cDNA segment of the SP-A1 allele 6A was cloned into an *Eco* RI site of the pEE14 vector. The 6A insert, in addition to the coding region, includes about 0.1 kb 5′ UTR and 0.1 kb 3′ UTR. Expression of the SP-A cDNA is driven by the human cytomegalovirus (CMV) immediate-early enhancer/promoter region. Recombinant DNA was performed according to standard methods (*42*). Transfection and selection were carried out according to the method described by Wang et al. (*37*).

Preparation of SP-A Variants and Native Human SP-A. SP-A was recovered and purified from the culture medium using mannose-affinity chromatography according to the method of Fornstedt and Porath (*43*). About 200 mL of serum-free culture medium (2×10^7 cells/plate/10 mL) were harvested from a 5-day culture and purified as described previously (*37*). Purified SP-A was concentrated using Amicon Centriprep-10 concentrators (Amicon, Beverly, MD). The native human SP-A was purified from BAL fluid obtained from alveolar proteinosis patients and healthy volunteers using a butanol-extraction method as described (*44*, *45*) with slight modification. In brief, after extraction of whole surfactant with butanol, the pellet was completely

dried with a flux of nitrogen gas and then homogenized twice in the buffer (20 mM *n*-octyl β-D-glucopyranoside, 10 mM Hepes, 150 mM NaCl, pH 7.4). After pelleting of the sample, insoluble protein was dissolved in 5 mM Tris/HCl, pH 7.4 and dialyzed for 48 h against the same buffer. The dialyzed solution was centrifuged at 155000x*g* at 4 °C for 30 min, and the supernatant containing SP-A was collected and kept at −80 °C. SP-A variants expressed from baculovirus-mediated insect cells were prepared according to the method as described (*39*).

All procedures were performed at 4 °C or on ice. Protein concentration was determined using the Micro-BCA method of Smith et al. (*46*) (Pierce, Rockford, IL) with RNase A as standard. SP-A was aliquoted and stored at −80 °C.

Gel Electrophoresis under Reduced, Nonreduced, and Native Conditions, Silver Staining, and Western Blotting. SDS−PAGE analysis was done following the procedure described by Laemmli (*47*). In vitro expressed SP-A variants and human SP-A from BAL fluid were subjected to electrophoresis under reducing, nonreducing, and native conditions. For reducing SDS−PAGE gels, SP-A samples were reduced with a loading buffer containing 0.1 M dithiothreitol (DTT), 1.0 M β-mercaptoethanol, 2% SDS, 0.1 M Tris/HCl (pH 6.8), and 10% glycerol, and heated at 95 °C for 10 min. The samples were subjected to electrophoresis on 10% polyacrylamide gels at 90 V for 3 h. For nonreducing SDS−PAGE, the SP-A samples were not reduced but mixed with a loading buffer containing 2% SDS, 0.1 M Tris/HCl (pH 6.8), and 10% glycerol, and heated at 95 °C for 10 min. The samples were subjected to electrophoresis on 4−15% acrylamide gradient gels at 90 V for 1 h and 70 V for 5 h. For native PAGE, the SP-A protein samples were prepared with a loading buffer (4×) containing 0.2 M Tris/HCl (pH, 7.5) and 40% glycerol and were not denatured by chemicals and heating. Electrophoresis was performed at 4 °C using 4−20% acrylamide gradient gels at 50 V for 1 h and 110 V for 17 h.

For silver staining of gels a modified version was used, as reported by Rabilloud (*48*). After color development, densities of the bands of oligomers on gels were quantified by soft laser densitometry. For Western blot analysis, proteins were transferred to PVDF membranes (0.2-μm pore size, Bio-Rad, Hercules, CA). SP-A was detected using a rabbit antibody (IgG) to human SP-A at a 1:1000 dilution (*39*). Then goat anti-rabbit IgG (HRP conjugated) antibodies were applied. Blots were exposed to XAR film following enhanced chemiluminescent detection.

MALDI-TOF Analysis of SP-A Variants. SP-A variants from the in vitro CHO cell expression system were analyzed using MALDI-TOF. About 8 μg of each sample were used for each MALDI-TOF run. Myoglobin (8 μg) was used as a positive control, and buffer alone was used as a negative control. SP-A samples and positive and negative controls were digested with endoproteinase Lys-C (Roche, Indianapolis, IN). Lys-C, a serine protease, hydrolyzes peptide bonds in proteins specifically at the carboxylic side of lysine (Lys). Before protease treatment, SP-A samples and controls were reduced and alkylated with iodoacetamide, and then digested with Lys-C at 37 °C overnight. The digestion was terminated by adding trifluoroacetic acid (TFA). Prior to MALDI-TOF analysis, the samples were desalted using C18-Zip Tips. Alpha-cyano hydroxycinnamic acid was used as the MALDI-TOF matrix and the MicroMass Tof Spec 2E machine for analysis. The results were calibrated with the MALDI-TOF four-point external calibration, and the peptide masses were obtained with the MALDI-TOF in the reflection mode. The theoretical mass of peptides from SP-A was estimated using a program online http://ca.expasy.org/cgi-bin/peptide-mass.pl.

Exposure of SP-A to Ozone and Detection of Protein Oxidation. SP-A proteins at a concentration of 1 mg/mL were exposed for 4 h to ozone (1 ppm) in 24-well tissue culture plates as described in our previous work (*37*, *49*).

Analysis of Amino Acids of Human SP-A Variants. Amino acid analysis following protein hydrolysis was done using the Aminoquant methodology (HPLC) with the recommended software, reagents, and protocol (Commonwealth Biotechnologies, Inc., Richmond, VA). To do analysis of different amino acids, several methods of hydrolysis were used. A hydrochloric acid hydrolysis method was used for general analysis of most SP-A amino acids. In brief, about 4 μg of pure protein were transferred to pyrolyzed glass tubes and dried. The samples were hydrolyzed in a gas phase 6 N HCl at 110 °C for 20 h. Following hydrolysis, the samples were again dried, dissolved in 50 μL of sample loading buffer, and 5 μL of the undiluted sample were analyzed. To determine the total tryptophan residues of the protein, about 6 μg of pure protein were transferred to a pyrolyzed glass tube and dried. The samples were hydrolyzed in 75 μL of 4 N methanesulfonic acid for 20 h at 110 °C. Following hydrolysis, the samples were neutralized (final volume 100 μL), and 5 μL of the undiluted sample was subjected to analysis. To determine oxidative products of methionine (Met) such as Met sulfoxide and Met sulfone, an alkaline hydrolysis method was used. About 12 μg of pure protein sample in 40 μL was added to 56 μL of fresh 4 N NaOH and 100 μL of water. The samples were mixed, loosely capped, and placed inside large glass test tubes. The tubes were constricted and sealed under vacuum. The samples were hydrolyzed for 15 h at 110 °C. Following hydrolysis, the samples were neutralized and the volume was recorded. Five microliters of each sample were subjected to analysis. To determine cysteine residues, about 5 μg of pure sample were placed in polypropylene tubes and taken to dryness. The samples were then dissolved in 300 μL of 6 M guanidine/HCl, 0.1 M Tris/HCl (pH 8.4) 5 μL (98%) of 2-mercaptoethanol were added and the sample was heated at 37 °C for 15 min. Then 2 μL of 4-vinylpyridine were added. The samples were heated for an additional 15 min and removed from the heat and an additional 1 μL of 4-vinylpyridine was added and the samples were allowed to stand for 2 h. The samples were dialyzed against diluted PBS and 0.05% SDS, dried, subjected to routine hydrolysis for 20 h, and subsequently dissolved in 50 μL of loading buffer. An aliquot (5 μL) of the undiluted sample was subjected to amino acid analysis. To determine which amino acids are oxidized and the relative number of oxidized residues, we calculated differences of a number of residues per SP-A molecule before and after ozone exposure. The assay described above for a given amino acid detects non-oxidized residues. Therefore, if a residue is oxidized the residue is not detectable by the assay, and the number of a given amino acid per SP-A molecule would be lower compared to that prior to ozone exposure. Moreover, the number of oxidized amino acids

per SP-A molecule was calculated based on the relative amount of each amino acid per SP-A molecule.

Protein Microsequencing of N-Terminal Amino Acid Sequences. The N-terminal amino acid sequence of SP-A variants was determined using modified Edman degradation method (Biotechnology Laboratory, UBC, Vancouver, Canada; and Midwest Analytical, Inc, St. Louis, MO). Pure protein (3 μg) was absorbed onto PVDF membranes for analysis. The relative percentage of each SP-A isoform was estimated by comparing the molecular yield of representative amino acids at the N-terminus of each sequence. Valine at position 22 was used for this calculation. For example, the number of molecules for sequences 1 and 2 starting with Glu^{21} and Cys^{20} (see amino acid terminal sequences in the results section) were determined from the sequencing information where valine was identified in position 2 of the Glu^{21} molecule and in position 3 of the Cys^{20} molecule. For N-terminal sequencing of each oligomer, pure protein was separated by electrophoresis on 4−15% polyacrylamide gradient gels using nonreducing conditions. The protein was transferred onto a PVDF membrane, and each band of oligomers was cut from the membrane for N-terminal sequencing.

ATP-Stimulated Phosphatidylcholine (PC) Secretion by Type II Cells. Alveolar type II cells were isolated from the lungs of pathogen-free young adult male Sprague−Dawley rats (approximately 225x*g) essentially according to the method of Dobbs et al. using digestion with elastase (50*), as previously described (*51*). The pneumocytes were plated on 35-mm plastic tissue-culture dishes (Costar, Cambridge, MA) at 3×10^6 cells/dish and cultured in 10% fetal calf serum (FCS) in Eagle's minimal essential medium (MEM) at 37 °C in a humidified incubator with 5% CO_2 in air for 24 h. Then 0.5 μCi/dish of (methyl-^{3}H)-choline (Amersham, Arlington Heights, IL) was added to the medium to label cellular phospholipids. To begin an experiment, cells were washed six times and incubated for 30 min in MEM. One set of cells was harvested at time 0 and served as control for phospholipid secretion associated with the medium change, as described previously (*52*). The remaining cells were preincubated with or without SP-A for 15 min, followed by addition of the secretagogue, adenosine triphosphate (ATP, 1 mM, Sigma, St. Louis, MO), to the media in a small volume. After 2 h, the medium was removed and centrifuged to remove detached cells. Methanol was added to the cell monolayer, and the cells were scraped from the dish. The cells and the media were extracted using the Bligh and Dyer method (*53*). The amount of phospholipid secretion was calculated as lipid degradations per minute (dpm) in the medium divided by the total dpm in lipid present in the cells plus the medium expressed as a %. All experiments were performed in duplicate, and the values were averaged. Results were analyzed statistically by one-way ANOVA or t-test using SigmaStat for Windows (Jandel, San Rafael, CA) where statistical significance is taken as $p < 0.05$.

RESULTS

Comparison of SP-A1 and SP-A2 Coding Sequences. We compared amino acid sequences of several human SP-A1 (6A, $6A^2$, $6A^3$, $6A^4$) and SP-A2 (1A, $1A^0$, $1A^1$, $1A^2$, $1A^3$) variants that occur with greater than 1% frequency (*17*). The alignment results of SP-A variants are summarized in Table 1. The percent similarities among SP-A2 variants varied from 98.8% (1A vs $1A^3$) to 99.6% ($1A^2$ vs 1A, or $1A^1$, $1A^0$ vs $1A^2$ or $1A^3$). The percent similarities among SP-A1 variants varied from 99.2% (6A vs $6A^4$, $6A^2$ vs $6A^4$ or 6A) and 99.6% ($6A^3$ vs $6A^4$ or $6A^2$ or 6A). The percent similarities among all SP-A1 and SP-A2 alleles varied from 96% ($1A^1$ vs $6A^4$) to 99.6% (several pairs), indicating that the $1A^1$ and $6A^4$ alleles are the most dissimilar alleles. Moreover, the data in Table 1 show that $6A^4$ is the most divergent SP-A1 allele compared to all SP-A2 alleles. After removal of the signal peptide (amino acids 1−20), the pairwise SP-A variants 6A and $6A^3$, $1A^0$ and $1A^2$, $1A^1$ and $1A^3$ are identical. Therefore, we used only six single SP-A gene variants 1A, $1A^0$, $1A^1$, 6A, $6A^2$, $6A^4$ and several coexpressed variants $1A^0/6A^2$, $1A^0/6A^4$, $1A^1/6A^2$ for further investigation. The rationale for the latter choice was based in part on previous findings where the $1A^0/6A^2$ haplotype is the most frequently observed in the population (*54*) and on the amino acid sequence comparison where the $6A^2$ and $6A^4$ are equally or less similar than other SP-A1 pair comparisons.

Table 1: Identity and Divergence Percentages of Human SP-A Variants Based on Amino Acid Sequence Comparison

	percent identity								
	1A	$1A^0$	$1A^1$	$1A^2$	$1A^3$	6A	$6A^2$	$6A^3$	$6A^4$
1A		99.2	99.2	99.6	98.8	97.6	97.6	97.2	96.8
$1A^0$	0.8		99.2	99.6	99.6	97.6	97.6	97.2	96.8
$1A^1$	0.8	0.8		99.6	99.6	96.8	96.8	96.4	96.0
$1A^2$	0.4	0.4	0.4		99.2	97.2	97.2	96.8	96.4
$1A^3$	1.2	0.4	0.4	0.8		97.2	97.2	96.8	96.4
6A	2.5	2.5	3.3	2.9	2.9		99.2	99.6	99.2
$6A^2$	2.5	2.5	3.3	2.9	2.9	0.8		99.6	99.2
$6A^3$	2.9	2.9	3.7	3.3	3.3	0.4	0.4		99.6
$6A^4$	3.3	3.3	4.1	3.7	3.7	0.8	0.8	0.4	
	percent divergence								

Characteristics of the Oligomerization of in Vitro Expressed SP-A Variants. In these experiments, we used purified SP-A protein from the conditioned medium of stably transfected CHO-K1 cell lines of six SP-A variants (6A, $6A^2$, $6A^4$, 1A, $1A^0$, $1A^1$) and two coexpressed SP-A variants ($1A^0/6A^2$, $1A^0/6A^4$). The yield of SP-A protein varied from 5 to 25 μg per 100 mL of conditioned culture medium.

First, we analyzed the eight SP-A variants under reducing conditions. SP-A protein was detected using silver staining of the gel and Western blot analysis with antibody against human SP-A after SDS−PAGE electrophoresis. The results in Figure 1A (silver staining) and 1B (immunoblotting) show that both the six single gene SP-A variants (1A, $1A^0$, $1A^1$, 6A, $6A^2$, $6A^4$) and the two coexpressed SP-A variants ($1A^0/6A^2$, $1A^0/6A^4$) have identical patterns in one-dimensional gel electrophoresis under reducing conditions. These consist of a major band (about 33 kDa) (monomers, 1×) and one less prominent band at about 66 kDa (dimers, 2×). Native human SP-A from BAL, which served as positive control, contained a major band and one less prominent band at the same positions. In addition, a band with very low intensity at about 100 kDa (trimers, 3×) in the native human SP-A was detected on the Western blot.

Second, we studied oligomerization of the in vitro expressed SP-A variants and native human SP-A under nonreducing conditions, where inter- and intramolecular disulfide bonds remain intact because no DTT, β-mercap-

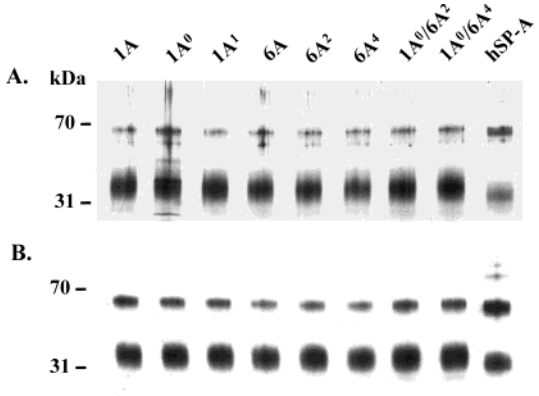

FIGURE 1: Gel electrophoresis of in vitro expressed SP-A variants and BAL-derived SP-A. SP-A variants were expressed from stably transfected CHO-K1 cell lines and were purified by mannose-affinity chromatography. SP-A from BAL of a healthy individual was prepared by a butanol-extraction method (*45*). Six single gene and two coexpressed SP-A variants as well as human SP-A (hSP-A) were reduced and separated by 12% SDS−PAGE under reducing conditions. Gels were subjected to silver staining (A) and Western blot analysis with IgG antibody to human SP-A (B). The numbers on left indicate molecular mass.

toethanol, or other sulfhydryl reducing agents were used. The patterns from the silver stained gel are shown in Figure 2A. The intensity of each band of oligomers was quantified by soft laser densitometry (Table 2). The results indicate that a markedly different set of oligomers exists for SP-A2, SP-A1, and coexpressed SP-A1/SP-A2. SP-A2 variants (1A, $1A^0$, $1A^1$) are characterized by four bands at about 66, 97, 130, and 200 kDa that may represent dimers (2×), trimers (3×), tetramers (4×), and hexamers (6×), respectively. These SP-A2 variants have dominant dimers (30−35%) and trimers (35−37%). SP-A1 variants (6A, $6A^2$, $6A^4$) consist of dimers (2×), trimers (3×), hexamers (6×), and multimers (>6×), but no tetramers (4×). SP-A1 variants have dominant trimers (32−36%) and hexamers (27−35%). Coexpressed SP-A variant $1A^0/6A^4$ has dimers (2×), trimers (3×), tetramers (4×), hexamers (6×), and multimers (>6×). The pattern of the coexpressed SP-A variants with regard to the oligomer size species was similar to those obtained with native human SP-A proteins (used as positive controls) from BAL fluid of alveolar proteinosis patients (AP hSP-A) and healthy volunteers (hSP-A). However, the amount of each oligomer species differed between the coexpressed and the SP-A from BAL (Figure 2A). AP hSP-A and hSP-A contain predominantly multimers (about 60−70% >6×), and the coexpressed SP-A variant ($1A^0/6A^4$) contains only 23.6% multimers (>6×).

Third, we studied oligomerization of SP-A variants and native human SP-As under native conditions as described in Methods. SP-A protein was not treated by SDS, DTT, β-mercaptoethanol, or heating. Therefore, SP-A molecules maintain their native conformation, and migration through a 4−20% gradient gel is based on the native molecular mass and structural molecular conformation. The results of the silver staining of the gel are shown in Figure 2B and the percentages of intensities of each band are summarized in Table 3. The patterns of oligomerization among SP-A1, SP-A2, and coexpressed SP-A1/SP-A2 differ. SP-A2 variants (1A, $1A^0$, $1A^1$) have, primarily, hexamers (6×) (50−68%), with dodecamers being the next abundant (12×) (25−35%),

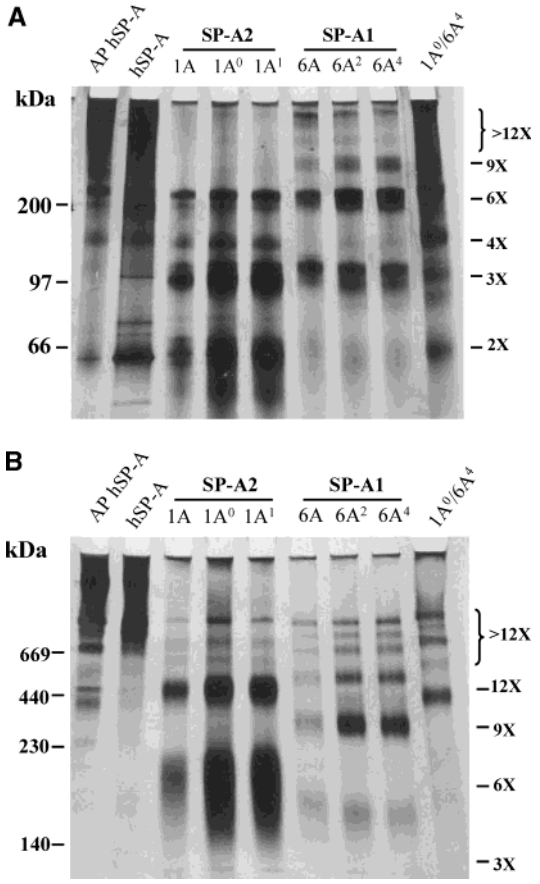

FIGURE 2: Patterns of oligomerization of SP-A variants and human SP-A's under nonreducing conditions (A) and native conditions (B). Six single gene SP-A variants (1A, $1A^0$, $1A^1$, 6A, $6A^2$, $6A^4$) and one coexpressed SP-A ($1A^0/6A^4$) were obtained from in vitro transfected CHO-K1 cell lines. Two human SP-A samples were prepared from bronchoalveolar lavage fluid of an alveolar proteinosis patient (AP hSP-A) and healthy volunteer (hSP-A), respectively. These samples were subjected to 4−15% SDS−PAGE under nonreducing conditions followed by silver staining (A) or were subjected to 4−20% PAGE under native conditions followed by silver staining (B). Numbers on left denote molecular mass. Marks on the right indicate oligomers.

Table 2: Quantification of Oligomers of Human SP-A Variants Separated by Nonreducing Gel Electrophoresis

	percentage (%) of oligomers				
human SP-A/SP-A variants	2X[a]	3X	4X	6X	>6X
AP hSP-A	7.8	4.1	8.7	14.7	64.8
hSP-A	20.2	5.0	7.6	9.5	57.7
1A	30.0	36.7	12.6	20.8	
$1A^0$	33.5	35.3	12.8	18.5	
$1A^1$	35.1	36.9	9.3	18.8	
6A	8.9	36.2		26.9	28.0
$6A^2$	5.7	35.1		33.7	25.5
$6A^4$	5.7	31.8		34.5	27.9
$1A^0/6A^4$	10.8	19.7	11.3	19.5	38.7

[a] Oligomers consisting of two (2X), three (3X), etc., monomers.

and multimers (>12×) with the lowest relative amount (7−15%). SP-A1 variants (6A, $6A^2$, $6A^4$) contain molecules in the range of hexamers (6×) (10−21%), dodecamers (12×) (15−21%), and multimers (>12×) (22−32%), with nonamers (9×) accounting for the highest relative amount (32−47%). Three bands larger than dodecamers (12×) are clearly visible in all SP-A variants, but it is difficult to identify their

Table 3: Quantification of Oligomers of Human SP-A Variants Separated by Native Gel Electrophoresis

	percentage (%) of oligomers			
human SP-A/SP-A variants	6X[a]	9X	12X	>12X
AP hSP-A			14.6	85.4
hSP-A			8.1	91.9
1A	50.6		34.5	14.9
$1A^0$	62.6		23.7	13.7
$1A^1$	68.2		24.8	7
6A	20.9	32.1	15.2	31.8
$6A^2$	14.2	46.8	17.2	21.8
$6A^4$	10.1	41.6	21.0	27.3
$1A^0/6A^4$			39.2	60.8

[a] Oligomers consisting of six (6X), nine (9X), etc., monomers.

exact degree of oligomerization due to the limitations of PAGE gels for macromolecule separation.

Identification of Coexpressed SP-A1/SP-A2 Products by RT-PCR and MALDI-TOF Analysis. SP-A1 and SP-A2 have very similar amino acid sequences, and antibodies specific to SP-A1 or SP-A2 are not available. To verify that both transfected SP-A1 and SP-A2 genes were expressed in the coexpressed clones, we examined specific SP-A1 and SP-A2 mRNA by RT-PCR and digestion of the RT-PCR products by gene-specific enzymes followed by gel electrophoresis. The results showed that both transfected SP-A1 and SP-A2 genes were expressed in the coexpressed clones (data not shown). Furthermore, we also determined SP-A1 and SP-A2 proteins by means of MALDI-TOF analysis (Figure 3). After digestion of the sample with endoproteinase Lys-C, which specifically hydrolyzes peptide bonds in proteins at the carboxylic side of lysine (Lys), the $1A^1$ variant produces a 3231.2 kDa peptide containing residues 224−248 (Figure 3), whereas the $6A^2$ produces a 3489.3 kDa peptide that contains residues 222−248. The $1A^1$ variant has a lysine at position 223, whereas the $6A^2$ has a glutamine at residue 223 and a lysine at 221. Figure 3 shows that both the 3231.2 kDa peptide and 3489.3 kDa peptide are present in the coexpressed $1A^1/6A^2$ variant. The RT-PCR data and MALDI-TOF data indicate that both gene products are expressed.

Characteristics of N-Terminal Sequences of SP-A Variants and Oligomers. The amino terminal sequences of four single gene products ($1A^0$, $1A^1$, $6A^2$, $6A^4$) and two coexpressed SP-A variants ($1A^0/6A^2$, $1A^0/6A^4$) were determined by protein microsequencing. The results are summarized in Table 4. All of the four single gene products have three amino terminal isoforms. The signal peptide cleavage sites are located between residues 18 and 19, 19 and 20, and 20 and 21 of the precursor molecule. The majority (about 80%) of these isoforms are cleaved between residues 19 and 20. Residue 19 of SP-A2 ($1A^0$, $1A^1$) and SP-A1 ($6A^2$, $6A^4$) is alanine and valine, respectively. Coexpressed SP-A variants of both SP-A1 and SP-A2 genes have four amino terminal isoforms and about half of them are products with the signal peptide being cleaved between residues 19 and 20. Moreover, two isoforms in which the first residue is alanine or valine were detected in coexpressed SP-A variants, further confirming that both SP-A1 and SP-A2 gene products are present in the coexpressed SP-A. In addition, the amino terminal sequences of two single gene products ($1A^0$ and $6A^2$) expressed in baculovirus-mediated insect cells were determined. The results indicated that there are some differences of N-terminal sequences between insect cell expressed SP-A variants and mammalian CHO-K1 cell expressed SP-A variants. For example, more than 95% of the SP-A2 ($1A^0$) molecules have Cys^{20} as their first amino acid, i.e., the signal peptide is cleaved between residues 19 and 20, whereas only about 50% of the SP-A1 ($6A^2$) molecules have Cys^{20} as their first amino acid, the other 50% of the SP-A1 ($6A^2$) molecules have Glu^{21} as their first amino acid.

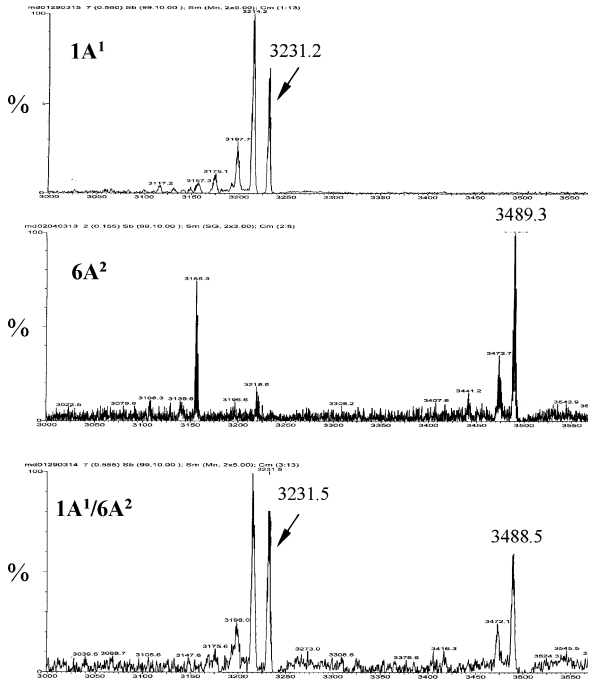

FIGURE 3: MALTI-TOF analysis of SP-A variants.Two single gene products $1A^1$ and $6A^2$ and coexpressed ($1A^1/6A^2$) variants were analyzed using MALDI-TOF. SP-A samples and controls were reduced and alkylated with iodoacetamide, and then digested with Lys-C at 37 °C overnight. The samples were analyzed and calibrated with the MALDI-TOF four-point external calibration. The single gene product $1A^1$ depicts a 3231.2 kDa peptide and the $6A^2$ variant depicts a 3489.3 kDa peptide. The coexpressed $1A^1/6A^2$ variant depicts both peaks of the 3231.2 kDa peptide and the 3489.3 kDa peptide demonstrating the presence of both gene ($1A^1$ and $6A^2$) products.

We next determined the amino terminal sequences of each oligomer (band) observed by gel electrophoresis at the nonreducing condition, using two representative SP-A2 ($1A^0$) and SP-A1 ($6A^2$) variants. The results are summarized in Table 5. About one-third of SP-A2 ($1A^0$) molecules present in dimers (2×) are of the shorter isoforms, i.e., first amino acid of N-terminal sequence is Glu^{21}, and the other two-thirds are of the middle size isoforms, i.e., first amino acid of N-terminal sequence is Cys^{20}. The other oligomers (3×, 4×, 6×) of SP-A2 ($1A^0$) have dominant middle size isoforms (i.e., Cys^{20}). The trimers of SP-A1 ($6A^2$) contain all three isoforms and other SP-A1 oligomers (6× and >6×) contain predominantly middle size isoforms (Cys^{20}) with one-third being the longer size isoforms (i.e., Val^{19}).

Characteristics of Ozone-induced Oxidation of SP-A Variants. To determine whether differences in SP-A properties exist between SP-A1 and SP-A2 variants following amino acid modification, we exposed SP-A variants (1A and 6A) to ozone at 1 ppm for 4 h. The optimal dose of ozone and time of exposure have been determined previously (*37*,

Table 4: Amino Terminal Sequence of in Vitro Expressed SP-A Variants[a]

	amino acid no.[b]									single gene products				coexpressed variants	
	19	20	21	22	23	24	25	26	27	$1A^0$	$1A^1$	$6A^2$	$6A^4$	$1A^0/6A^2$	$1A^0/6A^4$
sequence 1			Glu	Val	Lys	Asp	Val	Cys	Val	7%	5%	6%	4%	22%	23%
sequence 2		Cys	Glu	Val	Lys	Asp	Val	Cys	Val	81%	74%	78%	81%	55%	48%
sequence 3[c]	Ala	Cys	Glu	Val	Lys	Asp	Val	Cys	Val	12%	21%	X	X	15%	17%
sequence 4[c]	Val	Cys	Glu	Val	Lys	Asp	Val	Cys	Val	X	X	16%	15%	8%	11%

[a] The percentage of each isoform was calculated by the molecular yield (picomoles) of representative amino acid from each sequence divided by the molecular yield (picomole) of total isoforms (e.g., % sequence 1 isoform = pmol of sequence 1/pmol of total sequences). [b] Numbered according to the amino acid number of SP-A precursor. [c] The residue no. 19 of single gene products SP-A2 ($1A^0$, $1A^1$) and SP-A1 ($6A^2$, $6A^4$) is alanine (Ala) and valine (Val), respectively.

Table 5: Amino Terminal Sequence of Each Oligomer from SP-A Variants under Nonreduced Conditions[a]

	amino acid no.[b]									oligomers of $1A^0$				oligomers of $6A^2$		
	19	20	21	22	23	24	25	26	27	2X	3X	4X	6X	3X	6X	>6X
sequence 1			Glu	Val	Lys	Asp	Val	Cys	Val	28%				26%		
sequence 2		Cys	Glu	Val	Lys	Asp	Val	Cys	Val	72%	75%	>95%	>95%	65%	75%	71%
sequence 3[b]	Ala	Cys	Glu	Val	Lys	Asp	Val	Cys	Val		25%	<5%	<5%	X	X	X
sequence 4[c]	Val	Cys	Glu	Val	Lys	Asp	Val	Cys	Val	X	X	X	X	9%	25%	29%

[a] The percentage of each isoform was calculated by the molecular yield (picomoles) of representative amino acid from each sequence divided by the molecular yield (picomole) of total isoforms (e.g., % sequence 1 isoform = pmol of sequence 1/pmol of total sequences). [b] Numbered according to the amino acid number of SP-A precursor. [c] The residue no. 19 of single gene products SP-A2 ($1A^0$) and SP-A1 ($6A^2$) is alanine (Ala) and valine (Val), respectively.

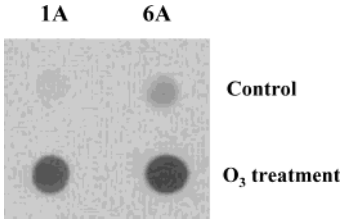

FIGURE 4: Ozone induced SP-A oxidation.Two representative SP-A variants, 1A and 6A, from SP-A2 and SP-A1, respectively, were exposed to ozone at 1 ppm for 4 h, which was proven to be optimal conditions for SP-A exposure in our previous studies (*37*, *49*). As a control, SP-A variants were exposed to filtered air for 4 h in the same apparatus (*37*, *49*). Each sample (200 ng) was blotted onto a membrane. and the oxidized proteins were detected using the OxyBlot method.

49), and SP-A oxidation was determined using the OxyBlot oxidized protein detection method. The results, in Figure 4, show that ozone-exposed SP-A variants (1A and 6A) are oxidized compared to unexposed SP-A variants. Coexpressed SP-A variants were also oxidized after ozone exposure (data not shown).

To determine which amino acids are oxidized and the relative number of oxidized residues of SP-A1 and SP-A2 variants, we calculated differences of a number of residues before and after ozone exposure. All amino acid residues of SP-A variants (1A and 6A) were analyzed using the Aminoquant methodology, and the number for each oxidized amino acid per SP-A molecule was calculated based on the relative amount of each amino acid per SP-A molecule. The findings for a single SP-A molecule (monomer) as well as the predicted values for native SP-A (18-mer or octadecamer) are shown in Table 6. For example, SP-A variant (1A) has three methionine (Met) residues (theoretical value) per SP-A molecule (monomer) based on the amino acid sequence of SP-A cDNA (*20*). The experimental values of Met are 2.8 and 1.7 (Table 6) before and after ozone exposure, respectively. The difference (1.1 Met) before and after ozone exposure represents oxidized Met residues. By extrapolating from the monomer values, we show the number of oxidized residues in native SP-A by multiplying the monomer values (difference before and after ozone treatment) by 18. For example, for the oxidized Met we observed 1.1 residues for the monomer, and for each native SP-A octadecamer we predicted about 20 Met oxidized residues (1.1×18) (Table 6). From the data in Table 6, we concluded that (i) a number of amino acids are oxidized after ozone exposure, including cysteine (Cys), histidine (His), lysine (Lys), methionine (Met), proline (Pro), serine (Ser), tryptophan (Trp), tyrosine (Tyr). (ii) Some differences in amino acid oxidation are observed between SP-A variants 1A and 6A. This may be the result of differences in their structure and/or conformation. (iii) Particularly interesting is the observation that both ozone-exposed SP-A variants (1A and 6A) increased by 0.5 and 0.6 of cystine per molecule, respectively, compared to unexposed SP-A variants. The additional cystine may affect SP-A molecular structure and conformation.

The oligomeric status of SP-A before and after ozone exposure was assessed by using nonreducing SDS−PAGE analysis. The results, in Figure 5, show that no additional bands are visible in either of the two single gene SP-A products ($1A^0$, $6A^2$) or coexpressed SP-A ($1A^0/6A^2$). However, the intensity of the bands of ozone-exposed SP-A variants is lower compared to those of unexposed SP-A variants, and this is particularly pronounced for $6A^2$. These findings may indicate that higher order multimers that could not enter the gel are formed in response to ozone.

Regulation of PC Secretion from Type II Cells by SP-A Variants. The relative ability of the SP-A variants to inhibit secretagogue-stimulated PC secretion is shown in Figure 6. SP-A from alveolar proteinosis patients and the SP-A alleles produced by Chinese hamster ovary (CHO)-K1 cells, SP-A1 ($6A^2$), SP-A2 ($1A^0$), and the coexpressed SP-A proteins SP-A1($6A^2$)/SP-A2($1A^0$), were added at 0.5 μg of SP-A protein/mL to ^{3}H-phosphatidylcholine (PC) labeled type II cells in the presence of 1 mM ATP. Since 1 μg of SP-A/mL

Table 6: Assay of Ozone-Induced Oxidation of SP-A Variants

	no. of residues/molecule									
			data observed by assay[b]				no. of oxidized residues[c]			
	data from sequence[a]		before O_3 treatment		after O_3 treatment		single SP-A (monomer)		native SP-A[d] (octadecamer)	
residue of SP-A	1A	6A	1A	6A	1A	6A	1A	6A	1A	6A
total Cys	7	8	6.8	8.2	6.2	7.9	0.6	0.3	11	5
cystine	ND	ND	2.7	2.8	3.2	3.4	0.5	0.6	9	11
His	4	4	3.7	3.2	3.3	2.8	0.4	0.4	7	7
Lys	7	7	7.1	9.1	7.1	8.3	0.0	0.8	0	14
Met	3	4	2.8	3.3	1.7	2.7	1.1	0.6	20	11
Met oxide	ND	ND	0.6	0.5	1.7	1.1	1.1	0.6	20	11
Pro	26	26	17.1	22.7	15.1	17.8	2.0	4.9	36	88
Trp	2	2	1.6	1.9	1.4	1.4	0.2	0.5	4	9
Tyr	8	8	6.1	6.3	5.7	6.0	0.4	0.3	7	5

[a] On the basis of published cDNA sequencing data (*20*). ND, not determined. [b] On the basis of amino acid analysis before and after ozone treatment. [c] The difference of residues before and after ozone treatment detected by assay; for native SP-A this difference is multiplied by 18. [d] For the purposes here native SP-A is noted as an octadecamer (i.e., equals to 18 SP-A molecules).

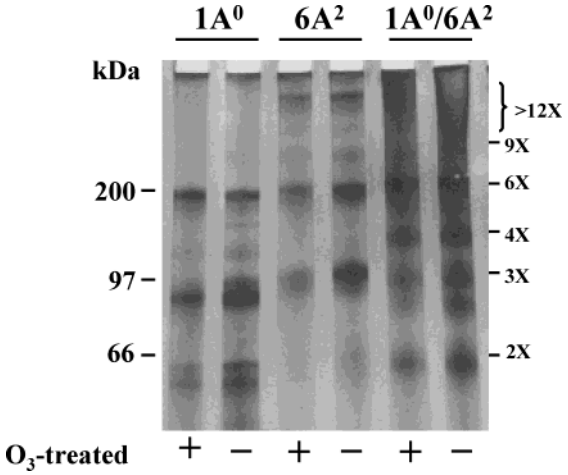

FIGURE 5: Patterns of oligomerization for SP-A variants before and after ozone exposure under nonreducing conditions. Two single gene SP-A variants ($1A^0$, $6A^2$) and one coexpressed SP-A ($1A^0$/$6A^2$) were exposed to ozone at 1 ppm for 4 h. Then the samples before and after ozone exposure were subjected to 4−15% SDS−PAGE under nonreducing conditions followed by silver staining. All bands of ozone-treated SP-A variants became weaker compared to those without treatment, but no additional bands were clearly visible. Numbers on left denote molecular mass. Marks on the right indicate oligomers.

produces maximum inhibition of PC secretion (*11*), comparisons were made at an SP-A concentration of one-half of maximum (0.5 μg/mL) to detect a greater or lesser activity of the alleles. Surfactant secretion into the media after a 2 h incubation period was measured and the data were expressed as a % of the PC secretion by type II cells treated with ATP (maximum PC secretion) alone. SP-A2 ($1A^0$) inhibited PC secretion to 29% of stimulated values, as did human SP-A (24% of maximum), as shown in Figure 6A. The SP-A1 variant ($6A^2$) significantly inhibited ATP-stimulated PC secretion but was not as effective as SP-A2. The SP-A1/SP-A2 protein, resulting from the coexpression of both gene products, blocked surfactant release to a similar extent as did human native SP-A and the SP-A2 allele, and to a greater degree than the SP-A1 allele ($p < 0.001$, $n = 3-6$).

Exposure of native SP-A to ozone has been shown to result in the oxidation of SP-A and modulation of SP-A function (*35*−*37*). To examine the effect of ozone on the biologic activity of SP-A alleles, we treated (*37*, *49*) the SP-A variants with 1 ppm ozone for 4 h at 37 °C and examined the ability

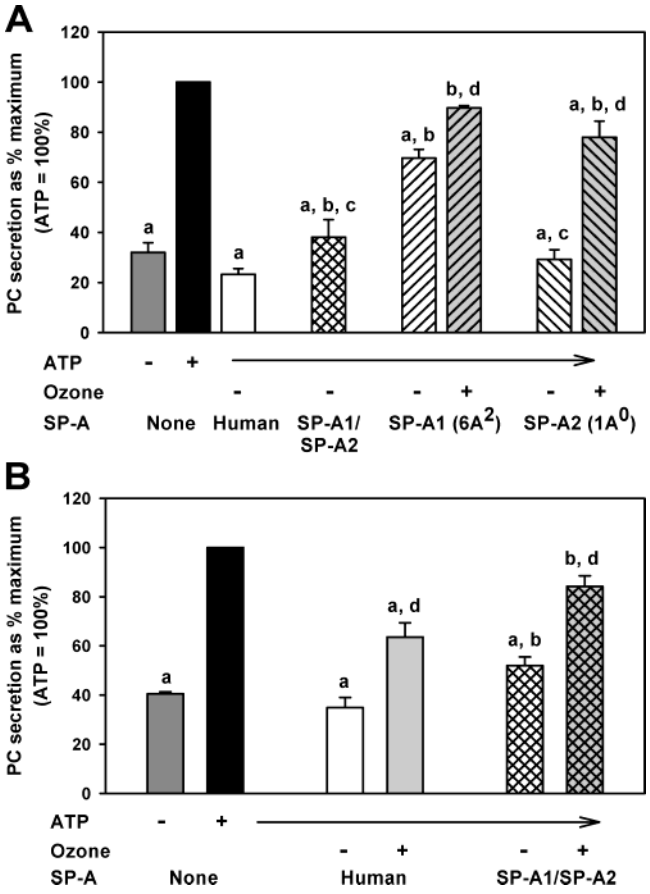

FIGURE 6: Differences in the biologic activity of SP-A alleles and the effects of ozone treatment. ^{3}H-PC-labeled type II cells were incubated without or with various SP-A species (0.5 μg SP-A/mL) for 15 min followed by no additions or ATP (1 mM) for an additional 2 h. Phospholipid secretion was determined as described in Methods and expressed relative to maximum secretion measured in the presence of ATP (=100%). SP-A alleles were expressed in vitro from CHO mammalian cells. ATP (1 mM) present in all samples except the first bar. Human SP-A, SP-A isolated from alveolar proteinosis patients. Ozone, SP-A exposed to ozone at 1 ppm for 4 h at 37 °C. Values are expressed relative to the maximum level of PC secretion as measured in the presence of ATP (=100%) and are means ± SE for $n = 3-6$. Basal PC secretion in the absence of additions = 1.7 ± 0.3%; PC secretion in the presence of ATP = 5.9 ± 0.9%, $n = 5-6$. Effects of ozone treatment on SP-A alleles individually (A) or in combination (B). (a) Significantly differed from ATP alone; (b) significantly different from human SP-A; (c) significantly different from SP-A1($6A^2$); (d) significantly different from no ozone treatment; all at $P < 0.05$.

of treated SP-A alleles to affect the phospholipid metabolism of type II cells. The detrimental effects of ozone on the ability of SP-A to stimulate cytokine production were evident after similar treatment conditions (*37*). Since the inhibition of PC secretion by SP-A variants exposed to air for 4 h at 37 °C did not significantly differ from untreated controls, the results from the two control groups were pooled. As shown in Figure 6A and B ozone treatment of the SP-A alleles and human SP-A reduced the ability of the proteins to prevent secretagogue-stimulated PC secretion. The partial effect of SP-A1 on surfactant release was completely abolished, as evidenced by the fact that ozone-treated SP-A1 ($6A^2$) no longer had a significant effect on ATP-stimulated PC secretion. The marked inhibition of PC secretion by SP-A2 was reversed significantly by ozone treatment ($p < 0.001$, $n = 3$), although the ozone-treated SP-A2 retained a partial ability to block secretion (Figure 6A). Coexpression of the two alleles (SP-A1/SP-A2) did not protect the proteins from ozone damage. Both the recombinant SP-A1/SP-A2 protein and the human SP-A were not as effective in reversing the effects of ATP on PC secretion after exposure to ozone (Figure 6B).

Recombinant SP-A variants expressed in the CHO-K1 cells more closely resemble SP-A isolated from human BAL fluid than variants expressed in Sf-9 insect cells. The mammalian cells are more capable of performing important posttranslational modifications to the SP-A protein (*37*). To determine whether the SP-A variants produced by insect cells were capable of inhibition of PC secretion, the biologic activity of the SP-A alleles produced in vitro in CHO cells were compared to those produced in Sf-9 cells. Increasing concentrations of all types of SP-A were incubated with ^{3}H-PC labeled type II cells together with 1 mM ATP and PC secretion was measured over 2 h. The SP-A2 and the SP-A1/SP-A2 variants produced in the CHO cells showed a similar biologic activity as human proteinosis SP-A at a low concentration (0.25 μg/mL) in the media (Figure 7A). Higher concentrations of these SP-A variants increased inhibition only slightly. All three inhibited ATP-stimulated PC secretion by 56−67%. The exception was SP-A1 which inhibited PC release by only 15% at a concentration of 0.25 μg/mL and required 1 μg/mL SP-A1 in the media for full inhibitory activity. The biologic activity of all of the SP-A variants produced in insect cells was not as effective as those produced in CHO cells and only partially inhibited PC secretion, even at an SP-A concentration of 1.5 μg/mL (Figure 7B).

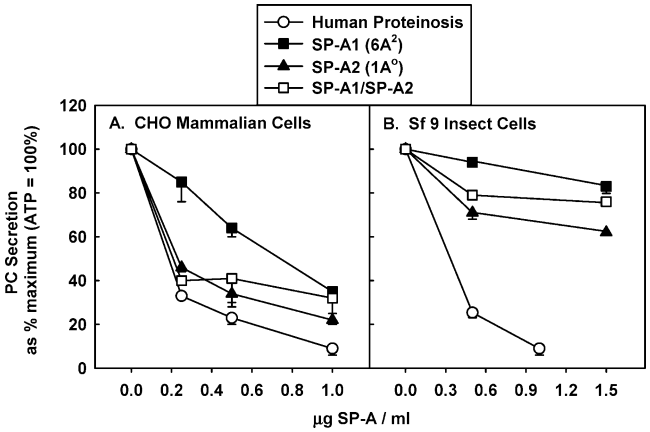

FIGURE 7: The effect of recombinant SP-A alleles on regulation of ATP-stimulated PC secretion by type II cells. ^{3}H-PC-labeled type II cells were incubated, harvested, and analyzed as in Figure 6. Data are from four experiments, each performed at one or two different SP-A concentrations. Each data point is the mean ± range of duplicate culture dishes. (A) SP-A alleles expressed in vitro from CHO mammalian cells. Basal PC secretion in the absence of additions = 1.5 ± 0.3%; PC secretion in the presence of ATP (set equal to 100%) = 4.4 ± 1.2%, $n = 3$. (B) SP-A alleles expressed in vitro from Sf-9 insect cells. PC secretion in the presence of ATP (set equal to 100%) was 5.0 ± 0.12 and 9.5 ± 0.3%, respectively (mean ± range), for two experiments performed in duplicate. Human proteinosis: SP-A isolated from alveolar proteinosis patients.

DISCUSSION

Human SP-A variants collectively differ in 10 amino acid residues including a cysteine. Single gene and both gene coexpressed SP-A variants stimulate TNF-α and IL-8 production by THP-1 cells. Differences in their ability to enhance proinflammatory cytokine production and in their susceptibility to ozone-induced oxidation have been observed among SP-A variants (*37*−*39*). However, the molecular/structural bases for the observed functional differences are not known. In this report, we characterized oligomerization patterns among SP-A variants and the impact of ozone-induced oxidation on the structure of SP-A variants. Several amino terminal sequence isoforms (three for single gene products and four for coexpressed) as well as differences in oligomerization exist among SP-A1, SP-A2, and coexpressed SP-A1/SP-A2 gene products. The majority of the isoforms contain a Cys^{20} residue that may be involved in disulfide bond formation. On the basis of quantification data of the amino terminal microsequencing of SP-A oligomers and observations of oligomerization patterns under nonreduced conditions, we put forward oligomerization models for SP-A1 and SP-A2 variants (Figure 8 see below). The extra cysteine of SP-A1 at position 85 appears to contribute to differences in the oligomerization patterns observed between SP-A1 and SP-A2 via the formation of inter-trimeric disulfide bonds. Ozone-induced oxidization of several amino acid residues including cysteine, tyrosine, methionine, and others may also contribute to SP-A structural differences (oligomerization and/or aggregation) that may result in alterations of SP-A function (this study) (*37*).

The majority of SP-A2 oligomers exist in the dimer and trimer forms, whereas the majority of SP-A1 are found in larger molecular forms (i.e., trimers and hexamers). SP-A is an oligomeric molecule, and some of its functions (if not all) are likely to depend on the status of its oligomerization, explaining perhaps some functional differences between SP-A1 and SP-A2 gene products (*37*, *39*, *55*). Recent findings indicated that the activity of surfactant protein D (SP-D) is dependent on its oligomeric structure in an in vivo mouse model (*56*). SP-D is a member of the collectin family and like SP-A plays a role in innate host defense. A study of isolated oligomers of the mannose-binding protein A (another member of the collectin family) showed that the larger oligomers can activate complement more efficiently (*57*). Moreover, association between human SP-A oligomer size and disease has been previously observed (*58*). The low molecular size forms of SP-A were increased in BAL of patients with birch pollen allergy compared to healthy controls. Some differences in oligomerization were also observed between AP-hSP-A and hSP-A under nonreducing

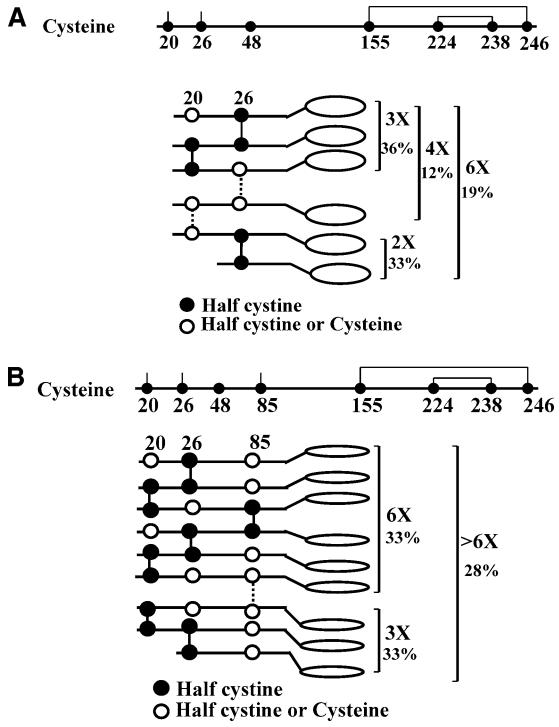

FIGURE 8: Models of oligomerization and intermolecular disulfide bonds for SP-A2 (A) and SP-A1 (B) variants. The cysteines shown previously (*28*) to form intramolecular (residues Cys^{155} and Cys^{246}, Cys^{224} and Cys^{238}) and interchain (Cys^{20}, Cys^{26}, and Cys^{85}) disulfide bonds are shown. Filled circles indicate half cystine, and open circles indicate residues that in some oligomers are half cystine and in others are cysteine. Solid lines (—) between two cysteines indicate intermolecular disulfide bonds, and dotted lines (- - -) indicate the formation or the absence of disulfide bonds. A dotted line that connects two Cys^{20} molecules (Figure 8A, SP-A2) can occur for all the amino terminal sequences except for sequence 1 (Table 4) that lacks Cys^{20} and its N-terminal amino acid is Glu^{21}. Also a dotted line that connects two Cys^{26} or two Cys^{85} molecules can occur in multimers ($\geq 3\times$) whereby at least one of their oligomers contains a molecule with a Glu^{21} N-terminal sequence (B, SP-A1). Dimers of SP-A2 and trimers of SP-A1 contain a Glu^{21} molecule(s), whereas multimers of SP-A2 and SP-A1 lack such molecules and have Cys^{20} and Ala/Val^{19} molecules. The percent of each oligomer is shown, as assessed under nonreducing gel electrophoresis conditions (Figure 2A) and their relative percentages as depicted in Table 2.

(Figure 2A) and native gel conditions (Figure 2B). Under the latter condition low molecular size forms, dodecamers ($12\times$), and pentadecamers ($15\times$), were found in theAP-hSP-A (from alveolar proteinosis patients), but not in hSP-A (from healthy individuals). The neck domain of SP-D appears to be necessary for assembly of trimeric subunits and dodecamers in vitro (*56*, *59*). Interestingly, although we attempted to express the CRD region of SP-A alone, we did not succeed in doing so without the presence of the neck region. Whether the lack of dimerization or trimerization (as may occur in the absence of the neck region) results in unstable SP-A monomers that are readily degraded remains to be determined.

We propose two models of disulfide bonds, each for SP-A2 and SP-A1 (Figure 8, panels A and B, respectively), based on the following data: (a) The pattern of SP-A oligomerization (Figure 2; Tables 2 and 3); (b) the amino terminal sequence analysis of SP-A isoforms (Table 4); (c) the frequency of each oligomer species in SP-A2 and SP-A1 (Table 5); and our observation that SP-A variants and hSP-A also contain free thiols as determined by amino acid analysis (Table 6) and DNTB (5,5′-dithiobis(2-nitrobenzoicacid)) assay (data not shown). Supported in part by the available data, the assumptions for the SP-A2 model, in Figure 8A, are (i) one-third of the molecules in the dimers lack the first cysteine (Cys^{20}) at the amino terminal sequence; a disulfide bond between Cys^{26}—Cys^{26} forms a dimer; (ii) disulfide bonds between Cys^{26}—Cys^{26} and Cys^{20}—Cys^{20} form a trimer ($3\times$), a tetramer ($4\times$), a hexamer ($6\times$), all of which contain Cys^{20} (Figure 2 and Table 5). SP-A1 variants (Figure 8B) have an extra cysteine at position 85. We suggest that this cysteine (Cys^{85}) forms a inter-trimeric disulfide bond between trimers and generates hexamers ($6\times$) and multimers ($>6\times$) identified under nonreducing conditions (Figure 2). Approximately, one-fourth of the molecules in the trimers lack the first cysteine (Cys^{20}) at the amino terminal sequence (Table 5). The assumptions of SP-A1 or SP-A2 molecules regarding the lack of Cys^{20} were made to account for the different amounts of amino terminal isoforms based on data shown in Table 5.

The amino terminal sequencing indicates that all of the single gene products and the coexpressed SP-A variants exist in several isoforms. This may result from an alternative cleavage site of the signal peptide, or other mechanisms, such as the removal of single amino acids following signal peptide cleavage (*60*, *61*). It was originally suggested (*62*) that the signal peptide cleavage of SP-A1 and SP-A2 occurred between residues 20 and 21 of the SP-A precursor. The PSIGNAL program of PC/gene predicts that potential cleavage sites for all SP-A2 variants and SP-A1 6A variant are between residues 20 and 21, 19 and 20, and 18 and 19; however, for the SP-A1, $6A^2$ and $6A^4$, the predicted cleavage sites are between 20 and 21, 18 and 19, and 17 and 18. Experimental analysis of the N-terminal amino acid identified isoforms resulting from all the predicted cleavage sites except the SP-A1 cleavage site between 17 and 18. However, both SP-A1 and SP-A2 variants were found with three N-terminal amino acid isoforms (Table 4) between residues 20 and 21, 19 and 20, and 18 and 19. All SP-A1 and SP-A2 variants have a dominant isoform with the first amino acid being Cys^{20} and two other isoforms, one with the first amino acid being Glu^{21} and the other with the first amino acid being an Ala^{19} (SP-A2) or Val^{19} (SP-A1). The dominant Cys^{20} isoform was also found in human SP-A's produced by the insect cell expression system, although differences in the frequency of Cys^{20} between SP-A1 ($6A^2$) and SP-A2 ($1A^0$) were observed (this study) (*55*). We speculate that the apparent discrepancy between predicted and actual N-terminal isoforms is explained by the involvement of mechanisms, such as hydrolysis of amino acids at the amino terminus. When we analyzed the N-terminal sequences of the intracellular $6A^4$ variant, the findings were similar to those obtained for the secreted protein (data not shown). This supports the notion that removal of the signal peptide between 20 and 21, 19 and 20, and 18 and 19 involves processes of cleavage and/or amino acid hydrolysis that occur intracellularly. The amino terminal sequencing analysis of each oligomer also indicates that different isoforms exist among different oligomers. The isoform that lacks Cys^{20} is not present in the higher size oligomers, suggesting that Cys^{20} plays an important role in the formation of different size oligomers. Moreover, it has been reported that rat SP-A protein isoforms arise by novel

mechanisms that include alternative translation initiation (*24*). However, in human SP-A translation starts only at one site,although another ATG (Met) codon is located at amino acid 13 after the translation initiation site (*20*).

Ozone can impair lung function and cause lung injury and inflammation via oxidative reactions with epithelial cells and surfactant components in the distal airspaces and airways of the lung. A remarkably high variability in response to ozone has been observed (*10*, *63*−*65*), and genetic factors have been implicated in this variability. Oxidized native SP-A exhibits changes in its molecular conformation and shows a decreased ability to interact with alveolar macrophages (*36*), to stimulate cytokine production by THP-1 cells (*37*), and to inhibit PC secretion by type II cells (*35*). In the present study, ozone treatment of the SP-A variants significantly interfered with the biologic activity of both SP-A1 and SP-A2 by blunting the ability of the proteins to inhibit surfactant secretion from alveolar type II cells to one-third of the values shown by untreated control SP-A forms. Differences in ozone susceptibility among human SP-A variants have been reported (*37*, *38*). The current results revealed that ozone oxidized several residues of SP-A variants including cysteine. Differences, in residue oxidation, between SP-A1 and SP-A2 variants were also observed, and these may result in differences of secondary structure and/or conformation. Ozone-induced SP-A aggregation may result from the formation of inter- and/or intramolecular disulfide bond(s) due to cysteine oxidation or from the formation of diotyrosine, due to tyrosine oxidation (*66*). This may lead to changes in SP-A on its structure (including oligomer formation) and function. Because no additional bands were observed in the SP-A pattern under nonreducing conditions after ozone treatment (Figure 5), the formation of new disulfide bonds in cysteine may be random among the various oligomers. These new oligomeric molecules may migrate at many different positions in the gel that cannot be detected either because their band intensity is too low, they migrate along with existing oligomer forms, or they are too large to enter the gel. Because the intensity of the bands after ozone exposure was uniformly decreased in the present study, our data favor the latter possibility. This possibility is consistent with recent findings of a simplified SP-A system of truncated human SP-A variants that lacked the collagen domain (*38*). These truncated SP-A variants showed higher size bands and aggregation following ozone exposure.

SP-A has the capacity to markedly modulate surfactant secretion from pneumocytes (*67*). The ability of SP-A to bind to the cell surface receptor on type II cells may be directly related to the effectiveness of the surfactant protein in the inhibition of secretagogue-stimulated surfactant secretion (*8*−*12*). Since SP-A variants have demonstrated structural and functional diversity, we determined the biologic activity of the SP-A alleles by measuring their interaction with type II cells and the resultant effect on PC secretion. We found that SP-A variants produced in CHO cells have the capacity to inhibit ATP-stimulated surfactant lipid secretion, although they exhibited differences in potency. SP-A2 and the coexpressed SP-A1/SP-A2 were the most effective at inhibiting secretagogue-stimulated surfactant secretion, followed by SP-A1, consistent with the possibility that the latter has a lower affinity for the SP-A receptor. The SP-A gene products produced in insect cells were not as effective as those from mammalian cells, suggesting that posttranslational protein modifications that occur in mammalian cells contribute to this SP-A function.

The parameters critical for the observed differences between SP-A1 and SP-A2 biologic activity are not clear. However, it is of interest to note that SP-A consisting of coexpressed SP-A2 and SP-A1 demonstrated the same biologic activity as SP-A2 alone, indicating that SP-A1 was not interfering with the properties of SP-A2 nor diluting its potency. While the carbohydrate recognition domain is felt to be critical for the interaction between SP-A and the SP-A receptor, there are no differences in the amino acid composition between SP-A1 ($6A^2$) and SP-A2 ($1A^0$) in that region of the protein. Although the human SP-A variants show differences in patterns of oligomerization, the formation of multimers was not found to be essential for the biological activity of rat SP-A expressed in an insect cell line (*68*) nor for the activity of the human SP-A alleles as demonstrated in the present report. In fact, the native gels of the human recombinant SP-A showed that the SP-A2 allele has the lowest amount of multimeric forms ($>12\times$), followed by SP-A1, and the coexpressed SP-A1/SP-A2, and finally by hSP-A with the highest multimeric form. However, SP-A1 was the least potent in the regulation of PC secretion from alveolar type II cells, while the other three SP-A forms demonstrated similar biologic activity. Nevertheless, the oligomeric status of the human surfactant protein in the presence of SDS may provide a partial answer for the differential functional activity. All of the most biologically active forms of SP-A, native hSP-A, SP-A2, and coexpressed SP-A1/SP-A2 contained the dimeric form of SP-A in the nonreducing gels containing SDS, while the dimeric form was much reduced in SP-A1. Whether the dimeric forms can access the SP-A receptor more readily than the larger size SP-A oligomers and thus their higher functional activity is unknown. Alternatively, formation of disulfide bonds due to the cysteines in position 85 of SP-A1 may make the protein more rigid or less stable (*55*), and these, respectively, may compromise movement of the carbohydrate recognition domain, or limit interaction of the protein with its receptor.

In summary, our results indicate that molecular, structural, oligomeric, and functional differences exist between SP-A1 and SP-A2. The extra cysteine of SP-A1 at position 85 may be involved in the formation of SP-A inter-trimeric disulfide bond, and account in part for differences in the oligomerization pattern between SP-A1 and SP-A2 variants that may reflect functional differences. Ozone-induced oxidation affected several residues including cysteine and induced aggregation to a varying degree for SP-A1 and SP-A2 variants. The changes described among SP-A1 and SP-A2 variants may in part reflect the molecular basis of functional differences observed under normal (*37*, *39*, *55*) and perturbed (i.e., following ozone exposure) (*37*, *38*) conditions. The SP-A1 variant was not as potent as the SP-A2 gene product in the regulation of secretagogue-stimulated surfactant secretion by pneumocytes. Therefore, the present data support qualitative differences between SP-A1 and SP-A2 gene variants. Moreover, in published reports variability in the expression of SP-A mRNA among unrelated individuals (*69*, *70*) and quantitative differences among SP-A1 and SP-A2 variants (*69*, *71*) have been observed. Together, these findings (*11*, *37*−*39*, *55*, *69*, *71*) suggest that in the human lung qualitative

and quantitative differences exist among SP-A's. It is possible that the SP-A alleles under certain conditions play a modifying role in the health or disease status of an individual.

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