Human SP-A genetic variants and bleomycin-induced cytokine production by THP-1 cells: effect of ozone-induced SP-A oxidation

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Huang, Weixiong, Guirong Wang, David S. Phelps, Hamid Al-Mondhiry, and Joanna Floros. Human SP-A genetic variants and bleomycin-induced cytokine production by THP-1 cells: effect of ozone-induced SP-A oxidation. Am J Physiol Lung Cell Mol Physiol 286: L546–L553, 2004.—Surfactant protein A (SP-A) plays a role in innate host defense. Human SP-A is encoded by two functional genes (SP-A1 and SP-A2), and several alleles have been characterized for each gene. We assessed the effect of in vitro expressed human SP-A genetic variants, on TNF-α and IL-8 production by THP-1 cells in the presence of bleomycin, either before or after ozone-induced oxidation of the variants. The oligomerization of SP-A variants was also examined. We found 1) cytokine levels induced by SP-A2 (1A, 1Aα) were significantly higher than those by SP-A1 (6A2, 6A4) in the presence of bleomycin. 2) In the presence of bleomycin, ozone-induced oxidation significantly decreased the ability of 1A and 1A/6A2, but not of 6A4, to stimulate TNF-α production. 3) The synergistic effect of bleomycin/SP-A, either before or after oxidation, can be inhibited to the level of bleomycin alone by surfactant lipids. 4) Differences in oligomerization were also observed between SP-A1 and SP-A2. The results indicate that differences among SP-A variants may partly explain the individual variability of pulmonary complications observed during bleomycin chemotherapy and/or in an environment that may promote protein oxidation.

Inflammation; enzyme-linked immunosorbent assay; oligomerization; ozone

PULMONARY SURFACTANT, which is produced by alveolar type II cells and consists of surfactant lipids and several associated proteins, is essential for normal lung function because it prevents alveolar collapse during end expiration (7). Pulmonary surfactant-associated protein A (SP-A), in addition to surfactant-related function (14), plays a role in innate host defense and regulation of inflammatory processes in the lung (8). It has been previously reported that natural human SP-A stimulates cytokine production (17, 28), nitric oxide production (2), alveolar macrophage phagocytosis (52), immune cell proliferation (27), NF-κB activation (25), and matrix metalloproteinase-9 production (53). In vivo studies suggest a role for SP-A in neutrophil recruitment in the lung of preterm lambs (26). However, in other systems, an anti-inflammatory role has been attributed to SP-A (1, 3, 4, 32). Some in vivo and in vitro studies show SP-A inhibition of LPS-induced cytokine production, nitric oxide, and lymphocyte proliferation. The reasons for these apparent discrepancies are not clear, but differences in the experimental systems are likely to contribute. Because surfactant lipids can moderate the proinflammatory effect of SP-A in vitro (25, 27, 28), it has been proposed that surfactant lipids and SP-A are counterregulatory. Therefore, changes in the relative amounts of surfactant lipids to SP-A may be important in determining the immune status of the lung. It is also possible that the role of SP-A changes at different stages of the inflammatory response and is modulated by different activating molecules (15, 47), as has been proposed recently for NF-κB (30).

Human SP-A is encoded by two functional genes (SP-A1 and SP-A2) and several alleles have been characterized for each gene based on nucleotide differences within the coding region. The most commonly observed alleles are 6A, 6A2, 6A3, and 6A4 for SP-A1 and 1A, 1A0, 1A1, 1A2, 1A3, and 1A5 for SP-A2 (10, 24). Several studies have shown differences among human SP-A genetic variants expressed in vitro in their abilities to stimulate cytokine production (55, 56) to bind carbohydrate (35) and lipids (16) and to enhance phagocytosis by alveolar macrophages (our unpublished observations). Moreover, a number of studies have revealed quantitative associations between SP-A alleles or genotype and mRNA levels, and different ratios of SP-A1 to SP-A2 mRNA have been observed among individuals (23). Associations between certain SP-A alleles and patient groups indicate that functional and/or structural differences of SP-A may play a role in the pathogenesis of certain diseases (13, 42).

Bleomycin is an effective chemotherapeutic agent and is used in the treatment of squamous cell carcinoma, lymphomas testicular carcinoma, etc. However, bleomycin-induced pulmonary fibrosis is a serious problem that limits the usefulness of the drug in some patients (49). Bleomycin induces inflammatory cells to secrete multifunctional cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-8, and transforming growth factor-β. These cytokines stimulate fibroblast proliferation and are probably involved in the development of pulmonary fibrosis (44, 57). It has been observed that bleomycin specifically stimulates macrophage-like THP-1 cells to produce TNF-α, IL-8, and IL-1β in a time- and dose-dependent pattern (22). In a clinical study, the TNF-α level was shown to be significantly increased after bleomycin infusion (46). TNF-α is considered to be a central mediator in bleomycin-induced pulmonary fibrosis, and TNF-α receptor knockout mice are protected from lung injury after exposure to bleomycin (38). Although the mechanism of bleomycin-induced toxicity has not been fully elucidated, it has been demonstrated that bleomycin-induced DNA damage is associated with reactive oxygen species. It is postulated that bleomycin exerts its...
toxic effects in the lung through the generation of oxygen free radicals that initiate pulmonary fibrosis (20). Clinical data have implicated oxygen supplementation during bleomycin chemotherapy with increased bleomycin toxicity (31).

Lung surfactant lines the surface of lung alveoli and is likely to be the first target of reactive oxygen species generated by oxygen and ozone (40). The effects of oxidants on the pulmonary surfactant system include the oxidation of surfactant proteins (including SP-A) and the peroxidation of surfactant phospholipids (50). Moreover, a number of studies have shown that oxidation of SP-A in vitro by ozone (36, 37, 56), nitrogen dioxide (34), and reactive oxygen and nitrogen species (9, 19) impairs many aspects of SP-A function, including lipid aggregation, mannos binding capacity, stimulation of cytokine production, and phagocytosis. In vivo oxidation of SP-A in different systems (34, 37, 48) has also resulted in functional differences in SP-A.

Our previous studies showed that SP-A enhances bleomycin-induced inflammatory cytokine production and that this synergistic effect can be attenuated by surfactant lipid (22). In the present study, we used the surfactant protein genetic variants as a model to gain insight into the basis of individual variability to bleomycin-induced pulmonary toxicity and specifically in microenvironments that may promote protein oxidation. In this regard, we assessed 1) differences among human SP-A genetic variants, expressed in vitro by insect cells either before or after ozone-induced oxidation, in their ability to stimulate cytokine production in the presence of bleomycin; and 2) whether functional differences correlate with differences in the oligomeric patterns of normal and ozone-oxidized SP-A variants under various electrophoretic conditions. The rationale for these studies is twofold. First, the functional and biochemical properties of SP-A change following ozone exposure (36, 37). In this regard, we hypothesized that the functional ability of SP-A variants is differentially affected by ozone-induced damage/oxidation. This differential alteration is due to amino acid differences among alleles. The second reason is that differences in SP-A oligomer size has already been associated with disease (21). Therefore, it is possible that ozone-induced alterations, as assessed by changes in the electrophoretic mobility, correlate with functional differences.

**MATERIALS AND METHODS**

**Cell lines and cell culture conditions.** The insect cell line SF9 (GIBCO-BRL, Life Technologies) from Spodoptera frugiperda was used for protein expression. The cells were cultured in SF-900 II SFM medium (GIBCO-BRL) in an incubator at 27°C. Protein expression proceeded in suspension culture of cells in a 250-ml Erlenmeyer flask (50 ml culture medium/flask) with shaking at 110 rpm.

The THP-1 cell line was obtained from the American Type Culture Collection (Manassas, VA). Cells were grown in RPMI 1640 (Sigma, St. Louis, MO) with 0.05 mM 2-mercaptoethanol and 10% heat-inactivated fetal bovine serum (BioWhittaker, Walkersville, MD) at 37°C in an atmosphere of 5% CO₂. The cells were split periodically and used at passages 8–15 in the various experiments. After differentiation with 10⁻⁸ M vitamin D₃ for 72 h, cells were washed with cold PBS. The cell pellet was resuspended in medium at a density of 2 × 10⁶ cells/ml in 24-well culture plates and exposed to bleomycin and SP-A. We terminated incubations by pelleting the cells. Supernatants were harvested and stored at −80°C until assayed.

**Preparation of native human SP-A.** Native human SP-A was purified from bronchoalveolar lavage (BAL) fluid of an alveolar proteinosis patient by the butanol extraction method as described in our previous report (22). The purified protein was examined by two-dimensional gel electrophoresis followed by Western blotting and silver staining and was found to be >98% pure. Western blot analysis was done with a rabbit anti-human SP-A IgG. For silver staining of gels, a modified version was used, as reported by Rabilloud (41). Protein concentration was determined with the micro-bicinchoninic acid method (Pierce, Rockford, IL) with RNase A as a standard. LPS content was determined with the QCL-1000 Limulus amebocyte lysate kit (BioWhittaker). This test indicated that human SP-A used in this study contained <0.01 pg LPS/μg SP-A. SP-A was stored at −80°C until use.

**Expression of human SP-A genetic variants by insect cells.** The SP-A genetic variants were expressed by the baculovirus-mediated insect cell system as described in our previous study (55). Briefly, PCR-amplified cDNA fragment was cloned into donor plasmid pFast-Bac DUAL (GIBCO-BRL). The recombinant plasmid was then transformed into Escherichia coli DH10Bac in which the foreign gene was transferred into the baculovirus genome (bacmid). After cloning, selection, and purification, the recombinant bacmid DNA was transfected into insect cell line Sf9. Three days after transfection, SP-A expression was examined by Western blot analysis, and recombinant baculovirus particles in the supernatant of the cell culture were collected. We achieved the expression of single SP-A gene products by infecting insect cells with the virus particles containing a single SP-A allele. A 50-ml culture at 2 × 10⁶ cells/ml was infected with 1 ml of a viral stock at 1 × 10⁹ pfu/ml. For coexpression of SP-A1/SP-A2 products, the virus particles containing SP-A1 and SP-A 2 genes were mixed at a 1:1 ratio of virus titers and used for infecting the insect cells. To confirm expression of the specific gene and/or allele, we determined the SP-A genotype of the insect cell mRNA by the PCR-converted restriction fragment length polymorphism (CRFLP) method (10). The culture supernatants were harvested at 72 h after infection and purified with mannose-affinity chromatography. The purified SP-A variants were then dialyzed against 5 mM Tris (pH 7.5) and examined by Western blotting and silver staining. Protein concentration and LPS content were determined by the methods described above. The LPS content of SP-A variants used in this study was <0.2 pg/μg SP-A.

**Bleomycin.** Bleomycin (Blenoxane; Bristol-Myers Squibb, Princeton, NJ) solutions were prepared immediately before use with endotoxin-free saline (American Pharmaceutical Partners, Los Angeles, CA). LPS was not detected in the stock solution of bleomycin at a bleomycin concentration of 5 U/ml (1 U = 1 mg) using the method described above.

**Electrophoretic analysis of in vitro expressed SP-A variants and native human SP-A under various conditions.** Human SP-A genetic variants and human SP-A from BAL fluid were subjected to electrophoresis under reducing, nonreducing, and native conditions. Reducing PAGE analysis was done following the procedure described by Laemmli (29). Electrophoresis (90 V, 1.5 h) of each protein sample was performed in a 10% polyacrylamide gel in the presence of SDS after reduction of protein by dithiothreitol and heating at 95°C for 10 min. The nonreducing PAGE (75 V, 5 h) was done in a 4–15% gradient polyacrylamide gel under the same conditions as the reducing PAGE, except there was no reducing agent in the loading buffer. Gel electrophoresis under native conditions was performed in a 4–15% gradient polyacrylamide gel with an electrophoretic running buffer (90 mM Tris, 80 mM boric acid, and Na₂EDTA, pH 8.4) and a loading buffer without reducing agent and SDS. In addition, electrophoresis was carried out at 4°C at 50 V for 1 h followed by 95 V for 13 h.

**Stimulation of THP-1 cells with SP-A and bleomycin.** After differentiation with 10⁻⁸ M vitamin D₃ for 72 h, THP-1 cells were pelleted and resuspended in cold PBS. Then, THP-1 cells at 5 × 10⁵/ml in RPMI 1640 at a density of 2 × 10⁶ cells/ml and incubated in 24-well culture plates (0.5 ml/well). Cells were stimulated with noozone or ozone-exposed SP-A (20 μg/ml), bleomycin (5 μU/ml), or SP-A plus bleomycin.
some experiments, Infasurf (Forest Pharmaceuticals, St. Louis, MO), an extract of natural surfactant from calf lung, was used to assess the inhibitory effect of surfactant lipid on SP-A, either with or without ozone-induced oxidation, and bleomycin on induced cytokine production. Cells were incubated for 4 h after treatment. TNF-α and IL-8 levels in culture medium were then quantified by ELISA.

**ELISA assay.** The ELISA assays for TNF-α and IL-8 (OptEIA Human ELISA Sets; Pharmingen, San Diego, CA) were performed according to the instructions recommended by the manufacturer. The ELISA kits were capable of measuring levels of 7.8–500 pg/ml for TNF-α and 6.2–400 pg/ml for IL-8. We obtained a reference curve for each of these cytokines by plotting the concentration of several dilutions of standard protein versus the corresponding absorbance.

**Exposure of SP-A to ozone and detection of protein oxidation.** SP-A protein at a concentration of ∼0.5 mg/ml was exposed to ozone in 24-well tissue culture plates as described previously (51). Briefly, each well contained 100 μl of the protein solution and was exposed to ozone (1 ppm for 4 h) for protein oxidation. Protein oxidation was detected with the OxyBlot oxidized protein detection kit (Intergen, Purchase, NY). The ozone-exposed proteins were derivatized with 2,4-dinitrophenylhydrazine (DNPH), 200 ng of DNPH-derivatized protein were blotted onto nitrocellulose, and immunodetection of oxidized proteins was done with anti-DNPH and goat anti-rabbit IgG (horseradish peroxidase-conjugated) antibodies. Blots were exposed to XAR film following enhanced chemiluminescent detection.

**Statistics.** Values are presented as means ± SE. Data were analyzed using SigmaStat statistical software (SPSS, Chicago, IL). For comparison among SP-A variants, statistical treatment included a one-way analysis of variance followed by a Student-Newman-Keuls test for pairwise comparison. A paired t-test was used for comparing the effect of SP-A before and after ozone-induced oxidation exposure. A value at P < 0.05 was judged to be significantly different.

**RESULTS**

Differentiated THP-1 cells were stimulated with SP-A variant (20 μg/ml), bleomycin (5 μM/ml), or SP-A variant plus bleomycin. In this study, in vitro expressed genetic variants of two SP-A1 alleles (6A2, 6A4), two SP-A2 alleles (1A, 1A0) that are commonly observed in the general population (12), as well as two combinations of SP-A1 and SP-A2 alleles (1A/6A2, 1A/6A4) were used. For the SP-A1/SP-A2 combinations we used a ratio of 1:1 viral SP-A1 and SP-A2 titers as noted in MATERIALS AND METHODS. Although it has been proposed that SP-A consists of a 2:1 ratio of SP-A1 to SP-A2 (54), the ratio of SP-A1 to SP-A2 at the mRNA level among unrelated individuals varies considerably (23). Therefore, it remains to be determined whether there is a unique ratio of SP-A1 to SP-A2 that applies to all individuals. A dose of 20 μg/ml of SP-A was chosen rather than the dose of 50 μg/ml we have used in previous experiments (55, 56), because the lower dose more effectively shows the combined effects of bleomycin and SP-A before or after ozone-induced oxidation. The doses of bleomycin and ozone used in this study were chosen based on findings of our previous studies (22, 56) and on other published reports (36, 44).

**Combined effect of in vitro expressed SP-A variants and bleomycin on cytokine production by THP-1 cells.** As depicted in Fig. 1A, TNF-α levels induced by SP-A1 variants (6A2, 6A4) are significantly lower than those by SP-A2 variants (1A, 1A0) and by coexpressed variants (1A/6A2, 1A/6A4). When these variants were combined with bleomycin, a synergistic effect on TNF-α level was observed for all SP-A variants. In the presence of bleomycin, 6A2 and 6A4 stimulated production of TNF-α by THP-1 cells to levels of 441 ± 34.9 pg/ml and 432 ± 64.9 pg/ml, respectively, which are significantly lower than those induced by 1A (635 ± 91.5 pg/ml) and 1A0 (603.8 ± 49.5 pg/ml). When IL-8 production was studied after the cells were treated with SP-A variants and bleomycin, the response pattern of IL-8 was very similar to that observed in TNF-α assay (Fig. 1B). No significant difference was found between SP-A2 and coexpressed variants in their ability to stimulate cytokine production in the presence of bleomycin.

**Effect of ozone-induced oxidation on SP-A/bleomycin-induced TNF-α production.** Functional changes in SP-A following ozone-induced oxidation are shown in Fig. 2. TNF-α
production was significantly reduced after ozone-induced oxidation of SP-A (99.0 ± 16.2 vs. 48.5 ± 5.8 pg/ml for 1A, 45.8 ± 2.8 vs. 33.0 ± 2.1 pg/ml for 6A*, 93.3 ± 7.1 vs. 47.1 ± 2.7 pg/ml for 1A/6A*). The synergistic effect of SP-A plus bleomycin on TNF-α production was also significantly decreased with oxidized 1A and 1A/6A* variants but not with the 6A* variant. When differences among SP-A variants in their ability to stimulate cytokine production before and after SP-A oxidation were compared, it was found that the TNF-α levels induced by 6A* were significantly lower than those induced by 1A or 1A/6A* in all circumstances except in the presence of bleomycin. Enhancement of TNF-α production by native human SP-A was also reduced after in vitro oxidation both in the presence and absence of bleomycin.

**Effect of surfactant lipids (Infasurf) on oxidized SP-A-induced TNF-α production by THP-1 cells in the presence of bleomycin.** The inhibitory effect of surfactant lipids (Infasurf) on native human SP-A plus bleomycin-induced TNF-α production was examined, both before and after ozone-induced oxidation of SP-A. Significant findings were observed in both the presence and absence of bleomycin (Fig. 3) either before or after SP-A oxidation.

**Patterns of oligomerization of in vitro expressed SP-A variants and human SP-A under various electrophoretic conditions.** To study whether the functional differences observed above were related to structural differences among SP-A variants, we analyzed in vitro expressed SP-A variants by PAGE under reducing, nonreducing, and native conditions. Under reducing conditions, the SP-A variants showed a monomeric form with a lower molecular mass than native human SP-A by both Western blot analysis (Fig. 4A) and silver staining (Fig. 4B) due to the absence of certain posttranslational modifications in the baculovirus-mediated expression system. A low-intensity dimeric band (~60 kDa) of SP-A variants was observed sometimes, especially by Western blot. The SP-A1 (6A*, 6A*) presented with a larger molecular mass than the SP-A2 (1A, 1A*) variants. The native human SP-A presented with both monomeric and dimeric forms under the reducing condition.

Under nonreducing conditions, the inter- and intramolecular disulfide bonds remain intact because no reducing agents, such as dithiothreitol and 2-mercaptoethanol, are used. The majority of oligomers of the in vitro expressed SP-A variants under this condition (Fig. 5A) are resolved into dimers (2X) and trimers...
highly oligomerized and most of them are 3X. In contrast, the oligomers of native human SP-A are native hSP-A under nonreducing (Fig. 5A). Patterns of oligomerization of in vitro expressed SP-A variants and native hSP-A by ozone exposure (Fig. 6A). Although oxidation of native human SP-A was detected before ozone exposure (Fig. 6A), a higher level of oxidation was observed after ozone exposure as assessed by a shorter film exposure (Fig. 6B). Densitometric analysis of two independent experiments performed in duplicate indicates that SP-A oxidation before ozone exposure is approximately (42 ± 4%) that following ozone exposure. Examination of native SP-A indicated that SP-A from the BAL fluid of patients with alveolar proteinosis is partially oxidized in vivo in the human body, and this has been shown previously (51).

Under reducing conditions, SP-A variants, but not native human SP-A, demonstrated bands of higher apparent molecular weight after ozone exposure (Fig. 7A). Although no significant loss of SP-A was detected by protein quantification after oxidation, bands of lower intensity in oxidized SP-A variants were observed in gel electrophoresis under nonreducing conditions (Fig. 7B). A major electrophoretic change in 6A4 was seen, compared with other SP-A variants after oxidation (Fig. 7, A and B). Increased smearing in the spaces between the oligomeric bands was observed in ozone-oxidized SP-A variants under nonreducing conditions (Fig. 7B). These observations point to the possibility that higher-size oligomers are formed and some of these may or may not enter the gel, explaining perhaps both the lighter band intensity and the smearing.

**DISCUSSION**

SP-A, a major surfactant-associated protein, plays important roles in surfactant-related activities and in the innate host defense of the lung. Due to its high level of heterogeneity and polymorphism, SP-A may contribute to the individual variability of susceptibility to pulmonary disease under certain conditions. The effect of bleomycin therapy on pulmonary toxicity may present an opportunity where the impact of SP-A variants could be studied. In the present study, we assessed the functional and structural differences among human SP-A genetic variants either before or after ozone-induced oxidation on ozone exposure at a concentration of 1 ppm for 4 h (Fig. 6A). Although oxidation of native human SP-A was detected before ozone exposure (Fig. 6A), a higher level of oxidation was observed after ozone exposure as assessed by a shorter film exposure (Fig. 6B). Densitometric analysis of two independent experiments performed in duplicate indicates that SP-A oxidation before ozone exposure is approximately (42 ± 4%) that following ozone exposure. Examination of native SP-A indicated that SP-A from the BAL fluid of patients with alveolar proteinosis is partially oxidized in vivo in the human body, and this has been shown previously (51).

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cytokine production by THP-1 cells in the presence of bleomycin, as well as the electrophoretic pattern of SP-A variants. The rationale for the latter is based on findings whereby SP-A oligomers of different sizes have been shown to associate with pulmonary disease (21). We found that the levels of cytokines induced by SP-A2 (1A, 1A0) were significantly higher than those by SP-A1 (6A2, 6A4) in the presence of bleomycin. Ozone-induced oxidation significantly decreased the ability of 1A and 1A/6A4, but not of 6A4, to stimulate TNF-α production in the presence of bleomycin. Moreover, the synergistic effect of bleomycin/SP-A either before or after oxidation was inhibited by surfactant lipids to the level of bleomycin alone. Structural differences in oligomerization were also observed between SP-A1 and SP-A2 variants, as assessed by gel analysis where changes were observed in both the molecular weight and the band intensity of SP-A variants following ozone-induced oxidation. These findings show that functional and structural differences exist among SP-A variants in the presence of bleomycin either before or after ozone-induced oxidation and indicate that structural differences may explain functional differences. The present findings also show that the ability of surfactant lipids to attenuate cytokine production is not influenced by the oxidation state of SP-A.

The high level of polymorphism of the SP-A genes may lead to quantitative and/or qualitative differences of the SP-A proteins among individuals, and this may contribute to the individual variability of susceptibility to pulmonary disease and bleomycin-induced pulmonary fibrosis. In the present study, we found that the ability of SP-A variants to enhance bleomycin-induced cytokine production is similar to that of native SP-A. However, differences with regards to the level of cytokine enhancement and the electrophoretic pattern between SP-A1 and SP-A2 variants were observed. Published data have shown the level of SP-A mRNA, as well as the ratio of SP-A1 to SP-A2 mRNA, to vary among individuals, indicating that, in some individuals, single SP-A gene products are in excess (23). An overabundance of one SP-A gene product over the other (23) may be reflected in functional differences among individuals, and such differences under certain compromised conditions may contribute to disease pathogenesis (11, 13, 42). Support for such a possibility is provided by two groups of experiments. First, functional differences between SP-A1 and SP-A2 have been observed not only by the findings in the present study but also by several in vitro studies. These include differences between the two SP-A gene products in cytokine production (55, 56), carbohydrate binding (16, 35), lipid aggregation (16, 35), structural stability (16), and phagocytosis (our unpublished observation). Second, association between certain SP-A alleles and patient groups has been observed (13, 42), suggesting that SP-A functional differences play a role in the pathogenesis of certain diseases. The present data, along with previously published reports, suggest that the SP-A1 and SP-A2 single gene products are not entirely equivalent in their functional capabilities at the protein concentrations studied.

A remarkable difference in the oligomeric pattern between SP-A1 (6A2, 6A4) and SP-A2 (1A, 1A0) was observed under various electrophoretic conditions. This may be of potential clinical interest in view of previous findings where patients with birch pollen allergy were identified with a larger fraction of smaller-size oligomers of SP-A (21). The difference in oligomeric patterns between SP-A1 and SP-A2 may relate to differences, among SP-As, in posttranslational modification (39), assembly (18), and thermal stability (16). For example, biochemical studies have shown: 1) the SP-A2 structure to be more stable than the SP-A1 structure and 2) differences in the ability of SP-A to undergo self-aggregation and to induce lipid and LPS aggregation, in order of native SP-A > SP-A2 > SP-A1/SP-A2 > SP-A1 (16). The higher structural stability of SP-A2 and/or of other structural differences between SP-A1 and SP-A2 may explain its higher capacity in cytokine production. In the present study, we did not observe a difference between the coexpressed variants and the single SP-A2 gene product, either in the function or in the oligomerization pattern, as we observed previously (16, 55). This is probably due to the variable ratio of SP-A1 to SP-A2 in the baculovirus-mediated expression system. In this system, the SP-A1/SP-A2 gene product was coexpressed by inculoting baculovirus containing both genes at a ratio of 1:1 (55). Although mRNA expression of both genes was confirmed by genotyping using PCR-cRFLP analysis (10), the ratio of SP-A1 to SP-A2 expression in each preparation may vary, and this variation may result in subtle differences in function and/or structure.

Bleomycin, which is used in several protocols of chemotherapy, is an exogenous lung oxidant and produces reactive oxygen species (20). In addition, patients who receive bleomycin therapy may need oxygen supplementation, and this hyper-

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**Fig. 7.** Electrophoretic analysis of in vitro expressed SP-A variants and native hSP-A after ozone exposure under reducing and nonreducing conditions. In vitro expressed hSP-A genetic variants (1A, 6A4, 1A/6A4) and native hSP-A purified from BAL of an alveolar proteinosis patient were exposed to ozone at a concentration of 1 ppm for 4 h. A: 1 μg protein was subjected to 10% polyacrylamide gel electrophoresis in the presence of SDS after reduction of protein in 50 mM dithiothreitol at 95°C for 10 min. B: 5 μg of each protein sample were subjected to a 4–15% gradient polyacrylamide gel electrophoresis in the presence of SDS after 95°C for 10 min without dithiothreitol where inter- and intramolecular disulfide bonds remain intact (nonreducing condition). Protein bands were detected by silver staining. Notation on the right (2X, 3X, etc) indicates oligomers.
oxia in turn may increase the oxidant burden in the lung via oxygen free radical production (31). A microenvironment with increased oxidative stress may promote oxidation of SP-A that in turn may alter its structure and/or its function. Oxidants such as ozone and nitrogen dioxide from air pollution contribute to surfactant phospholipid oxidation (40) and function, and thus these agents may also contribute to SP-A oxidation. In the present study, ozone-induced oxidation of SP-A resulted in a significant decrease in its synergistic effect with bleomycin to stimulate TNF-α production. The functional reduction of SP-A after ozone-induced oxidation is consistent with other in vitro and in vivo reports, where various oxidants including ozone were used to study aspects of SP-A function (9, 34, 36, 37).

Müller and coworkers (34) compared SP-A functions following in vivo and in vitro exposure of SP-A to nitrogen dioxide and found that the in vivo exposure was less effective in altering SP-A function with regard to its mannose binding capacity, protein-lipid aggregation, and lipid secretion. It is possible that under relatively normal conditions protective mechanisms, such as the presence of adequate levels of antioxidants, ions, and lipids, exist to prevent SP-A from being fully oxidized. Moreover, animal studies showed SP-A, but not SP-B and SP-C, to be increased following bleomycin treatment (43) and nitrogen dioxide treatment (34). We speculate that the increased amount of free SP-A in the presence of oxidant-producing molecules overloads the antioxidant system and further contributes to the bleomycin-induced proinflammatory effect. Alternatively, the increased amount of SP-A in bleomycin-treated animals may help alleviate bleomycin-induced surfactant dysfunction due to derangement of other surfactant components. For example, SP-A is capable of reversing the biophysical properties of oxidized surfactant lipids (6) and can inhibit lipid peroxidation (5). It is also possible that the ability of oxidized SP-A to protect surfactant lipids from peroxidation is diminished and the resulting oxidized surfactant lipids no longer attenuate the SP-A/bleomycin synergism. In this scenario a prolonged inflammatory response ensues that may lead to lung injury and fibrosis.

There was no significant change of the 6A4/bleomycin synergistic effect on TNF-α level after ozone-induced oxidation of 6A4 in the presence of bleomycin. This indicates that the response of 6A4 may differ from that of 1A and 1A/6A4 under the present experimental conditions. A tryptophan instead of an arginine at position 219 within the carbohydrate recognition domain that distinguishes the 6A4 from other frequently found SP-A1 alleles may contribute to this observation, although the finding is rather paradoxical because a tryptophan is more susceptible to ozone oxidation than an arginine (33). Of interest, the 6A4 allele has been shown to exhibit inferior capacity to induce LPS aggregation (16), enhance cytokine production (56), and be a risk factor in the pathogenesis of idiopathic pulmonary fibrosis (45). However, the mechanism as to how the 6A4 may contribute to these different responses and specifically whether the distinguishing amino acid (Trp) plays a role is unknown. It is possible that differences in structure and stability play a role in these processes. The electrophoretic data (Fig. 7) following ozone-induced oxidation show a more pronounced change in 6A4 compared with 1A and 1A/6A4, with a loss of higher-size oligomers.

In summary, with regard to cytokine production by macrophage-like cells in response to SP-A, we found 1) differences between the SP-A1 and SP-A2 single gene products in the presence of bleomycin, 2) a difference between the SP-A 6A4 variant and the other SP-A variants after ozone-induced oxidation in the presence of bleomycin, and 3) electrophoretic differences in the oligomeric band pattern between SP-A variants either before or after ozone-induced oxidation. We speculate that differences observed by gel analysis have an impact on the function of SP-A variants under unperturbed conditions and conditions that promote protein oxidation. We further speculate that a better understanding of the mechanisms that underlie the differences among SP-A variants may contribute to our better understanding of individual variability of pulmonary complications observed during bleomycin chemotherapy and/or in an environment with increased oxidative burden.

GRANTS

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