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ORIGINAL INVESTIGATION

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Surfactant protein A and B genetic variants predispose to idiopathic pulmonary fibrosis

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Abstract Derangement in pulmonary surfactant or its components and alveolar collapse are common findings in idiopathic pulmonary fibrosis (IPF). Surfactant proteins play important roles in innate host defense and normal function of the lung. We examined associations between IPF and genetic polymorphic variants of surfactant proteins, SP-A1, SP-A2, SP-B, SP-C, and SP-D. One SP-A1 ($6A^4$) allele and single nucleotide polymorphisms (SNPs) that characterize the $6A^4$ allele, and one SP-B (B1580_C) were found with higher frequency ($P \leq 0.01$) in nonsmoker and smoker IPF (n=84) subgroups, respectively, compared with healthy controls (n=194). To explore whether a tryptophan (present in $6A^4$) or an arginine (present in other SP-A1 alleles and in all SP-A2 alleles) at amino acid 219 alters protein behavior, two truncated proteins that varied only at amino acid 219 were oxidized by exposure to ozone. Differences in the absorption spectra (310–350 nm) between the two truncated recombinant SP-A proteins were observed both before and after protein oxidation, suggesting allele-specific aggregation differences attributable to amino acid 219. The SP-B SNP B1580_C (odds ratio:7.63; confidence interval:1.64–35.4; $P \leq 0.01$), to be a risk factor for IPF smokers, has also been shown to be a risk factor for other pulmonary diseases. The SP-C and SP-D SNPs and SP-B-linked microsatellite markers studied did not associate with IPF. These findings indicate that surfactant protein variants may serve as markers to identify subgroups of patients at risk, and we speculate that these contribute to IPF pathogenesis.

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Introduction

Pulmonary surfactant and surfactant proteins in general have been shown to play important roles in surfactant-related functions, such as the lowering of surface tension in the alveoli at low lung volumes (Floros and Phelps 1997), and in host-defense functions including the regulation of proinflammatory cytokine production, chemotaxis, and tissue repair (Floros and Phelps 2002; Phelps 2001). Therefore, derangement in composition, structure, or function of surfactant proteins (SP-A, SP-B, SP-C, SP-D) may contribute to the development of many pulmonary diseases.

The surfactant protein genes have all been characterized and found to have a number of genetic polymorphisms and/or mutations. These surfactant protein polymorphisms, although not lethal, may, under certain conditions, compromise health and contribute to a disease pathogenesis of complex etiology in subjects who carry specific polymorphic markers. Therefore, the physiological relevance of the surfactant proteins to lung health and their natural genetic variation, which can serve as "tags" to identify disease subgroups, make surfactant proteins good models in the study of pulmonary diseases.

Idiopathic pulmonary fibrosis (IPF), a progressive lung disorder of unknown etiology, is characterized by sequential acute lung microinjuries involving epithelial injury/activation with subsequent fibroblastic foci formation, scarring, and end-stage lung disease (Gross and Hunninghake 2001; Selman et al. 2001). The disease is usually lethal and mostly unresponsive to stereocorticoid/immunosuppressive therapy (Collard and King 2001; Selman et al. 2001). IPF is a complex disease in which genetic susceptibility is likely to be influenced by many genes acting in concert or independently, and by environmental factors that together determine the development of the disease. Until now, there have only been a few studies trying to identify susceptibility gene polymorphisms, and most of them have dealt with candidate genes related to cytokine pathways (Freeburn et al. 2001; Hutyrova et al. 2002; Pantelidis et al. 2001; Renzoni et al. 2000; Whyte et al. 2000).

The human SP-A locus consists of two functional genes, SP-A1 and SP-A2, with several polymorphisms that have been described within the coding region of the SP-A1 and SP-A2 genes and that result in amino acid substitutions (DiAngelo et al. 1999). Importantly, quantitative studies have shown that one homozygous SP-A1:SP-A2 genotype ($6A^2/6A^2$:$1A^0/1A^0$) is correlated with low to moderate level of gene expression (Karinch et al. 1997). Of interest, a recent study of human SP-A 3'-untranslated-region constructs has identified the $6A^4$ allele as having lower basal mRNA level compared with other variants (Wang et al. 2003b). Human SP-D is linked to the SP-A locus on 10q22-q23.1 (Hoover and Floros 1998), and at least two exonic polymorphisms have been described in SP-D (DiAngelo et al. 1999). Genes mapping to chromosomes 2p12-p11.2 (Vamvakopoulos et al. 1995) and 8p21 (Fisher et al. 1988) encode the hydrophobic proteins SP-B and SP-C, respectively. Several polymorphisms have been identified in the SP-B gene, and at least one of them may affect protein processing (Lin et al. 1998; Wang et al. 2003a). A highly polymorphic variable nucleotide tandem repeat region within intron 4 of the SP-B gene (Floros et al. 1995) and flanking SP-B-linked microsatellite polymorphic markers have also been identified (Kala et al. 1997). None of the surfactant protein genetic polymorphisms has been previously examined for their association with IPF. On the other hand, SP-C mutations have been associated with several forms of human familial idiopathic interstitial pneumonias (Nogee et al. 2001; Thomas et al. 2002).

In the present study, we have focused our attention on genetic polymorphisms rather than mutations of the surfactant proteins. Polymorphisms, although not lethal, are found frequently in the population (>1%) and may contribute to and/or modify disease outcomes. Specifically, we have investigated polymorphisms in SP-A, SP-B, SP-C, and SP-D in IPF patients, for the following reasons. (1) There is evidence indicating that epithelial damage and alveolar collapse may be critical in the pathogenesis of IPF, and changes in lung surfactant and biophysical surfactant function have been reported in this disease (Burkhardt 1989; Honda et al. 2002; Myers and Katzenstein 1988; Selman et al. 2001). (2) SP-A increases collagen expression by lung fibroblasts, and SP-A and SP-D influence the synthesis and secretion of matrix metalloproteinases by human alveolar macrophages and monocytic cells, indicating that SP-A and SP-D participate in extracellular turnover (Trask et al. 2001; Vazquez de Lara et al. 2000, 2003). (3) Bronchoalveolar lavage (BAL) levels of SP-A (Phelps et al. 2003) and serum levels of SP-A and SP-D are increased in IPF, and serum levels of SP-A and SP-D appear to predict survival. The estimated 5-year survival of IPF patients with low serum levels is considerably higher than those with high levels (Greene et al. 2002). Moreover, the serum levels of SP-D at the time of diagnosis may predict the velocity of decline in pulmonary function (Takahashi et al. 2000). The reasons for these findings are unknown but may reflect the severity of alveolar epithelial injury and increased leakage of SP-A and SP-D from the airspace to the vascular compartment. (4) SP-A and SP-D modulate a variety of immune cell functions and influence the proliferation of T cells and rat splenocytes (Borron et al. 2002; Kremlev et al. 1994). (5) SP-C mutations have been associated with familial idiopathic interstitial pneumonias (Nogee et al. 2001; Thomas et al. 2002). Therefore, because of the physiological relevance of the surfactant proteins to pulmonary disease, we deem them to be good candidates in the study of IPF and speculate that their genetic polymorphisms will help to identify subgroups at risk.

Patients and methods

Patients

Eighty-four unrelated IPF patients were included in this study (Table 1). Thirty were current or former smokers (35.7%), and 59 were male (70.2%). The protocol was approved by the Ethics Committee at the Instituto Nacional de Enfermedades Respiratorias, Mexico. Diagnosis of IPF was supported by clinical observation, pulmonary function, high-resolution computed tomography,

Table 1 Demographic and clinical characteristics of the IPF study group (*ND* not done)

Characteristic	IPF	Controls
Gender (male/female)	59/25	124/70
Age (years)	62.3±10.9	41±14.5
Nonsmokers/smokers	54/30	103/91
Duration of disease, months	27.4±18.3	
Clubbing	57/84	
FVC % predicted	62.6±14.6	106.5±11.3[b]
FEV_1 % predicted	65.6±15.8	99.7±12.8[b]
FEV_1/FVC%	88.0±10.1	79.3±5.5[b]
PaO_2 mm Hg[a]	51±9.3	ND
BAL macrophages %	78.7±7.2	ND
BAL lymphocytes %	14.4±6.8	ND
BAL neutrophils %	4.4±2.7	ND
BAL eosinophils %	2.5±1.9	ND

[a]Normal values at Mexico City altitude: 67±3 mm Hg
[b]Performed in 122 healthy controls

and BAL findings and was corroborated by surgical biopsy in 43 patients based on the typical morphology of normal interstitial pneumonia (Katzenstein and Myers 1998). In the absence of biopsy, patients had to fulfil the criteria of the American Thoracic Society and Eurpoean Respiratory Society international consensus (American Thoracic Society 2000). Patients with known causes of interstitial lung disease (i.e., collagen vascular disease, drug toxicity, environmental exposure) were excluded. The control group (n=194) comprised smoking (n=91) and nonsmoking (n=103) healthy male (n=124 or 63.9%) and female (n=70) volunteers with a mean age of 41±14.5 years. Nonsmokers were sequential unrelated healthy blood donors from the National Institute of Respiratory Diseases. Smokers were randomly recruited from the Smoking Cessation Program in the Institute. They had a smoking history for more than 5 years but displayed normal pulmonary function tests. Both IPF patients and controls consisted of individuals from families in which at least three generations had been born in Mexico.

Bronchoalveolar lavage

BAL was performed as previously described (Selman et al. 2000). Aliquots of the cell pellet were fixed in carbowax and stained with hematoxylin and eosin for differential cell counting.

Genotyping

Genotyping was performed by using genomic DNA as template. For single-nucleotide polymorphism (SNP) genotyping, we employed the polymerase chain reaction (PCR) and converted PCR restriction fragment length polymorphism (RFLP) analysis. This approach is based on the idea that if a single-base polymorphism does not contain a natural enzyme restriction site, it is converted to a restriction recognition site by using a primer with the appropriate mismatched nucleotide. Therefore, samples homozygous for the particular SNP will either be digested with the enzyme used or they will not be subject to digestion, resulting in either case in one visible band. However, samples heterozygous for the particular SNP will result in two bands upon agarose gel electrophoresis because only one allele will be cut, and the other will remain intact. The SP-A1 alleles (e.g., $6A^4$) are haplotypes, their designation being based on the pattern of five SNPs within the coding sequence. An example of this is discussed and shown below for the SP-A1, $6A^4$ allele. In the present paper, we studied all the frequently found SP-A1 (6A, $6A^2$, $6A^3$, $6A^4$) and SP-A2 (1A, $1A^0$, $1A^1$, $1A^2$, $1A^5$) alleles, plus some rare alleles. For the SP-A analysis, we considered both the alleles and the individual SNPs, and for the SP-B, SP-C, and SP-D polymorphisms, we considered the individual SNPs. Genomic DNA isolation and genotyping for SP-A, SP-D, and SP-B SNPs and SP-B-linked microsatellites were carried out as described previously (DiAngelo et al. 1999; Guo et al. 2001). The SNPs and the microsatellite markers studied have been described in detail previously (DiAngelo et al. 1999; Kala et al. 1997; Lin et al. 1998). The method for SP-C genotyping is as follows. Two SP-C SNPs, viz., CA138(A/C) and CA186(A/G), were identified by the direct sequencing of PCR products that were amplified from lung genomic DNA. The notation for these SNPs is as follows: the C (for example, in the CA138 SNP) denotes SP-C and the A138 or A186 in the CA186 denotes amino acid 138 or 186, respectively. The number of the amino acid sequence noted is that of the SP-C precursor prior to signal peptide cleavage. Both SNPs are missense mutations. The CA138(A/C) SNP is located at the 2nd nucleotide of amino acid 138, resulting in an amino acid change from Asn (AAT) to Thr (ACT). The CA186(A/G) SNP is located at the 2nd nucleotide of the amino acid 186, resulting in an amino acid change from Asn (AAC) to Ser (AGC).

The SP-C genotyping method is a PCR-based cRFLP analysis. Briefly, a 1279-bp PCR fragment was amplified with primers 1106 and 1107 from 100 ng genomic DNA. The fragment was used as a template for converted PCR with primers 1114 and 1115A for SNP CA138(A/C), and with primers 1108 and 1116 for SNP CA186(A/G). Primer 1106 is antisense: taggtgacactatagaatacAAATCAGGCTGCTTTATTCTG. Lower case letters denote non-SP-C sequences attached for other experimental purposes. Primer 1107 is sense: ACTGGCCTCGTGGTGTATGA; primer 1108 is sense: GATGGAATGCTCTCTGCAGG; primer 1114 is sense: GCTGATCGCCTACAAGCCAG; primer 1115A is antisense: CTGGAAGTTGTGGACTTTaCTA; primer 1116 is antisense: GCACCTCGCCACACAGGGaG. Underlined lower case letters denote a mismatched nucleotide. Primers 1115A and 1116 were modified, with each containing a mismatched nucleotide to convert CA138(A/C) to an *Spe*I and CA186(A/G) to a *Sac*I recognition site, respectively. The converted PCR products were digested with *Spe*I or *Sac*I (New England BioLabs). For CA138(A/C), allele C, and for CA186(A/G), allele G were cut by the appropriate enzyme. The digested PCR products were separated on 8% polyacrylamide gel electrophoresis (PAGE). The genotype was assigned based on the pattern of the digested PCR fragments amplified from different alleles.

Statistical methods

The analysis was performed as follows. First, by the use of logistic regression following adjustments for smoking and sex, each marker allele was tested to determine whether homozygosity had a different effect from heterozygosity. Because almost all of the tests were not significant, it was assumed that there was no significant dose effect. Thus, in the subsequent analyses, all markers were dichotomized into either the presence or absence of one particular allele. Specifically, for SNPs, the one allele resulting in the less significant P-value for testing the dose effect was chosen to be the baseline, and for the other polymorphic markers, the alleles not of interest were grouped to form the baseline. Next, we examined whether unbalanced frequencies existed between the case and the control groups for each allele. The corresponding allele and smoking interaction was also tested. A P-value of <0.1 indicated that associations between the marker allele and IPF may differ between smokers and nonsmokers. In this case, stratified analyses according to smoking status were performed. Analyses with Fisher's exact test were also performed to account for small cell counts for some alleles. Markers that showed significant differences in either the logistic regression or Fisher's exact test are presented and discussed below. To account for multiple testings, a more stringent significance level of 0.01 was used. To determine whether the Hardy-Weinburg Equilibrium (HWE) held for each of the marker loci within the control group, the HWE test was performed by using the computer program Genetic Data Analysis (Lewis and Zaykin 2001).

Preparation of truncated SP-A variants, ozone exposure, gel electrophoresis, and UV absorbance

Truncated SP-A alleles ($1A^{0t}$, $6A^{4t}$), containing the SP-A carbohydrate recognition domain (CRD) and neck domains (amino acids 100–248), and lacking the amino terminal and collagen regions, were cloned in pGia vector, and proteins were obtained by using *Escherichia coli* expression and the His-Bind purification system (Novagen, Madison, Wis.). The truncated $1A^{0t}$ is identical in amino acid sequence to the $6A^{4t}$ allele, except with respect to amino acid 219.

The SP-A, $1A^{0t}$, $6A^{4t}$ alleles were studied before and after oxidation. Oxidation was achieved by ozone exposure and confirmed by oxyblot analysis as described previously (Umstead et al. 2002; Wang et al. 2002). SP-A truncated protein variants, at a concentration of 1 mg/ml were oxidized by exposure to ozone in 24-well tissue culture plates at 1 ppm for 4 h. The protein variants were subjected to electrophoresis on a 8%–16% acrylamide gradient gel under non-reducing conditions to determine whether oxidation altered their electrophoretic mobility, and their absorbance at wavelengths ranging from 240 nm to 390 nm was determined by using a U-2001 spectrophotometer. Differences in absorbance may reflect differences in protein folding or aggregation properties.

Table 2 SP-A and SP-B genetic variants: IPF vs Controls; univariate analysis (*OR* odds ratio, *CI* confidence interval)

At least one copy of the given allele	Model[a]	Controls %	IPF %	OR	95% CI of OR	*P*-value	*P*-value for Fisher's exact test
SP-A1_6A^4	Nonsmokers	6.8 (7/103)	22.2 (12/54)	3.67	1.34–10.07	0.01	0.01
AA219_T	Nonsmokers	7.8 (8/103)	22.2 (12/54)	3.13	1.18–8.32	0.02	0.02
AA50_C	Nonsmokers	74.8 (77/103)	94.4 (51/54)	6.68	1.87–23.86	<0.01	<0.01
AA62_G	Nonsmokers	52.4 (54/103)	77.8 (42/54)	3.34	1.56–7.14	<0.01	<0.01
B1580_C	Smokers	67.0 (61/91)	93.3 (28/30)	7.63	1.64–35.4	0.01	<0.01

[a]In the logistic regression, if the *P*-value of the interaction term between smoking and marker allele was less than 0.1, indicating that smoking is an effect modifier for the disease and allele association, analyses were stratified by smoking status. For the model "Non-smokers", only non-smokers were used and for the model "Smokers", only smokers were used. All models were adjusted for sex. For the model "S+N", smoking was also adjusted for; no significant findings were observed for the model "S+N"

Results

Clinical characteristics of IPF study group

The baseline characteristics of the IPF patients are shown in Table 1. They presented with progressive dyspnea and cough (duration 27.4±18.3 months), predominantly basal inspiratory crackles, radiographic bilateral interstitial opacities, high resolution computed tomography scan patterns with varying degrees of reticular infiltrates, subpleural and basal honeycombing, and sparse ground-glass opacification. All patients showed significant reduction in lung volumes and hypoxemia at rest that worsened with exercise. BAL cell profile exhibited percentages of normal lymphocytes with a moderate increase in neutrophils and eosinophils.

Associations of SP-A, SP-B, SP-C, and SP-D variants with IPF

The significant findings ($P \leq 0.01$) for SP-A are shown in Table 2, and a schematic presentation of the SNPs that identify the SP-A1, 6A^4 allele, is shown in Fig. 1.

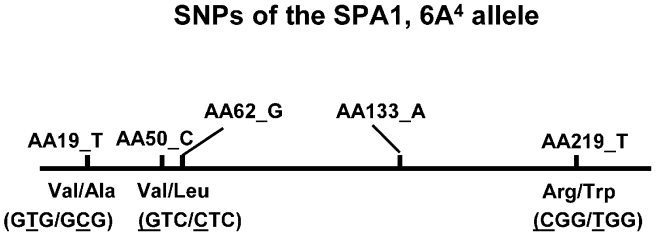

Fig. 1 Schematic representation of the single nucleotide polymorphisms (SNPs) for the SP-A1 (6A^4) alleles. All the SNPs that identify the 6A^4 allele are shown *above* the *line*. The notation AA19, AA50, etc. denotes the following: the first A in AA19 indicates that this SNP is for SP-A and the A19 notation indicates that the particular SNP is within the codon for amino acid 19. SNPs AA62 and AA133 do not change the encoded amino acid, but the other three do. The change for AA19 is Val/Ala corresponding to the GTG/GCG alleles, respectively; for AA50 is Val/Leu corresponding to GTC/CTC alleles; for AA219 is Arg/Trp corresponding to CGG/TGG

The SP-A1, 6A^4 allele and three (AA50_C, AA62_G, AA219_T) of the five SNPs (Fig. 1) that distinguish the 6A^4 allele from the other SP-A1 alleles appear, based on the odds ratios, to be risk factors for the nonsmoker subgroup only. Two of the three SNPs are significant ($P<0.01$), and the third (AA219_T) approaches significance ($P=0.02$; Table 2). One of the SP-B SNPs (B1580_C) appears to be a risk factor when smokers are considered alone (Table 2). No significant SP-B SNPs were observed when the nonsmoker subgroup was analyzed alone, or when both smokers and nonsmokers were considered together. Moreover, in the control group, all the marker loci studied (except AAGG) were in HWE (data not shown), indicating lack of stratification bias. In the AAGG case, the HWE was violated in both IPF and control groups.

Biochemical studies of 6A^4 allele

SP-A1 alleles are haplotypes and are classified based on the nucleotide pattern exhibited at five SNPs (DiAngelo et al. 1999). An example is shown in Fig. 1. Because several of the SNPs that change the encoded amino acid and distinguish the SP-A1 6A^4 allele from the other SP-A1 alleles, were found to be significant in the analyses performed above (Table 2, Fig. 1), we studied the 6A^4 allele further. We reasoned that amino acid differences between the 6A^4 and other SP-A1 alleles that have a differential impact on the structure and/or function of the SP-A1 alleles may, under certain conditions, contribute to disease pathogenesis. The first part of the speculation was tested partially by means of a simplified system, as described below, where we studied structural changes between the 6A^4 and other SP-A1 alleles before and after oxidation. Ozone was the agent used to cause SP-A oxidation. Protein oxidation is relevant to IPF, because the microenvironment in IPF is such that it may promote oxidation of proteins. For example, decreased total and reduced glutathione levels are found in the epithelial lining fluid in IPF (Beeh et al. 2002; Behr et al. 1995; Cantin et al. 1989), and these changes may be responsible for increased oxidation of proteins (Lenz et al. 1996).

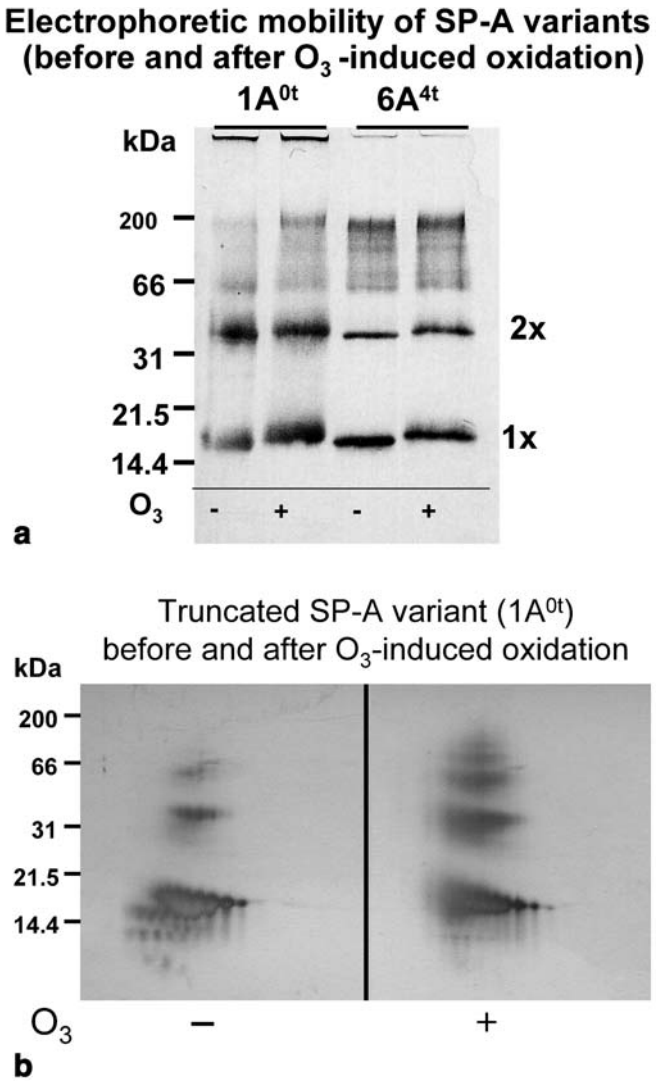

Fig. 2a, b Electrophoretic patterns for SP-A truncated variants before and after ozone exposure. The two truncated SP-A variants ($1A^{0t}$, $6A^{4t}$) were oxidized via exposure to ozone at 1 ppm for 4 h. The oxidized samples (+) and the non-oxidized (–) samples were subjected to 8%–16% SDS-PAGE under non-reducing conditions followed by silver staining (**a**) and to two-dimensional PAGE analysis (**b**). *Numbers left* (**a, b**) Molecular mass, *numbers right* (**a**) oligomers. Monomer (*1×* 17.4 kDa) and higher size oligomer (*2×*). A representative experiment out of three performed is shown in **a**, and the $1A^{0t}$ allele is shown in **b**, out of a total of three alleles studied with similar results

As shown in Fig. 1, $6A^4$ is characterized by three SNPs that change the encoded amino acid (AA19, AA50, and AA219). The amino acid changes at positions 19 and 50 are probably less important in the function and/or structure of the protein. In both cases, a small hydrophobic amino acid is substituted for another similar amino acid. However, the SNP at amino acid 219 results in a significant change. An arginine, a highly hydrophilic and charged amino acid is substituted for a tryptophan. As a result, we focused our attention on AA219 during further experimentation.

Differences at amino acid 219

To determine whether a Trp (present in $6A^4$ and $6A^5$) or an Arg (present in other SP-A1 alleles) at amino acid 219 alters properties of SP-A alleles, we performed biochemical analysis of two truncated constructs ($1A^{0t}$, $6A^{4t}$) that differ only at amino acid 219; one construct had Arg and the other had Trp. These truncated constructs allowed for a simplified system in which the contribution by factors, such as self-aggregation or oligomer formation attributable to the collagen domain, is eliminated. The SP-A truncated proteins were studied by electrophoresis on non-denaturing gels for changes in their electrophoretic mobilities (Fig. 2) and by absorption spectra (Fig. 3, Table 3) for differences in their aggregation properties. The SP-A truncated proteins were studied before and after ozone-induced oxidation.

Both $1A^{0t}$ and $6A^{4t}$ depicted similar patterns before and after oxidation. Both variants after oxidation exhibited lower electrophoretic mobility as assessed by one-dimensional gel electrophoresis (Fig. 2a) and an increase in aggregation/oligomerization accompanied by a decrease in the definition of isoforms as shown by two-dimensional PAGE analysis. An example is depicted in Fig. 2b in which the $1A^{0t}$ allele is shown before and after ozone oxidation. Similar results were obtained for the $6A^{4t}$ allele (not shown). Moreover, an increase in absorption at 310–350 nm and 240–260 nm (Fig. 3) was observed. These data together indicate that ozone-induced oxidation can directly affect the non-collagenous portion of the SP-A (neck and CRD domains). Differences in absorption prior to oxidation were observed in the range of 240 nm to

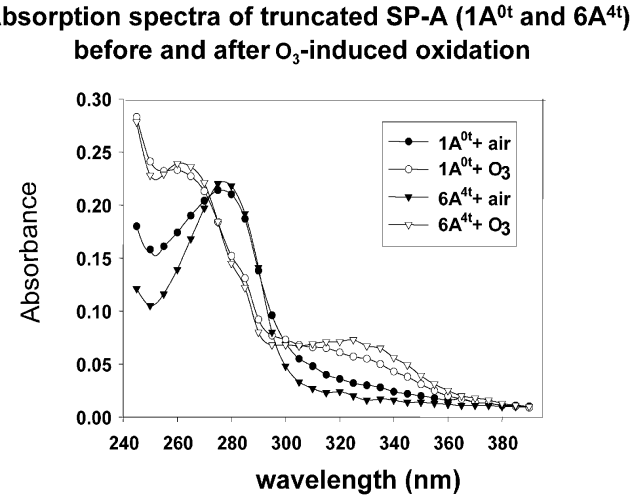

Fig. 3 Absorption spectra of truncated SP-A variants before and after ozone exposure. Two truncated SP-A variants ($1A^{0t}$ and $6A^{4t}$) were exposed to ozone at 1 ppm for 4 h. The absorbance of SP-A before and after ozone-induced oxidation was measured at the range of UV from 240 nm to 390 nm by using a U-2001 spectrophotometer. Differences in the absorbance at the range of 310 nm to 350 nm between the two SP-A variants both before and after oxidation reflect significant aggregation differences as also shown in Table 3. This figure depicts the mean values from three experiments

Table 3 Absorbance (*ABS*) of truncated SP-A variants ($1A^{0t}$ and $6A^{4t}$) before and after ozone-induced oxidation at a range of wavelengths (*NS* not statistically significant)

Wavelength (nm)	ABS of $1A^{0t}$			ABS of $6A^{4t}$		
	+ Air[a]	+ O_3	O_3 vs air (*P*-value)	+ Air	+ O_3	O_3 vs air (*P*-value)
300	0.070 ± 0.005	0.074 ± 0.004	NS	0.046 ± 0.002	0.067 ± 0.007	p<0.05
310	0.049 ± 0.003	0.069 ± 0.003	p<0.05	0.027 ± 0.004	0.069 ± 0.005	p<0.01
320	0.035 ± 0.004	0.060 ± 0.006	p<0.01	0.024 ± 0.005	0.071 ± 0.004	p<0.01
330	0.031 ± 0.006	0.055 ± 0.003	p<0.01	0.016 ± 0.005	0.068 ± 0.005	p<0.01
340	0.025 ± 0.007	0.044 ± 0.005	p<0.05	0.015 ± 0.003	0.056 ± 0.003	p<0.01
350	0.021 ± 0.004	0.032 ± 0.006	p=0.05	0.013 ± 0.004	0.041 ± 0.003	p<0.01
360	0.020 ± 0.003	0.026 ± 0.004	N.S.	0.015 ± 0.006	0.026 ± 0.007	N.S.

[a]The absorbance data shown are from three independent experiments and depict the Mean±SE

260 nm (Fig. 3). At this wavelength, the absorbance of certain amino acids including Trp ($6A^{4t}$) differs, and differences between the two alleles in this range may reflect differences in protein folding. However, protein oxidation eliminated differences in absorbance (240–260 nm) between the $1A^{0t}$ and the $6A^{4t}$ alleles (Fig. 3). In contrast, in the wavelength range of 310 nm to 350 nm, differences between the two alleles were observed both before and after oxidation. However, the increase in absorbance (310–350 nm) of $6A^{4t}$ after oxidation was more pronounced than that of the $1A^{0t}$ (Fig. 3, Table 3), even though the baseline level (i.e., before oxidation) was lower for the $6A^{4t}$ allele than for the $1A^{0t}$ allele. These observations indicate that the self-aggregation properties of the truncated alleles differ both before and after ozone-induced oxidation. Oxidation of these alleles appears to affect the biochemical properties of SP-A, and this, in turn, may under certain conditions affect function. Because the two truncated alleles shown here differ only at amino acid 219, this amino acid probably plays a differential role in the structural and/or functional properties of SP-A.

Discussion

Surfactant proteins play important roles in the maintenance of normal alveolar function and in host defense of the lung. Derangements in normal lung function and/or host defense are central to several pulmonary diseases. Therefore, quantitative and/or qualitative differences in the surfactant proteins probably contribute to the pathogenesis of pulmonary disease. Moreover, the natural genetic variability of these proteins may help identify subgroups at risk for a particular disease entity or for pulmonary disease in general. To evaluate the latter possibility, we examined associations of IPF with SP-A, SP-B, SP-C, and SP-D polymorphisms. Our results show that SP-A, and SP-B SNPs appear to confer disease susceptibility. However, no association was found for the SP-C or SP-D SNPs or the SP-B flanking microsatellite marker alleles studied here.

The SP-B allele (B1580_C), shown with increased frequency in IPF smokers, has been revealed to be also a risk factor for chronic obstructive pulmonary disease, indicating that this allele may be an important variant in pulmonary disease pathogenesis in smokers (Guo et al. 2001). The SP-B SNP (B1580) marks an important change. We have observed that the SP-B allele (B1580_C), which differs from the T allele (B1580_T) at amino acid 131 (C=Thr; T=Ile), creates a potential site for N-linked glycosylation (Lin et al. 1998). Recently, we have demonstrated that this potential glycosylation site of the C allele is indeed glycosylated at the Asn129-Gln-Thr131 site, whereas the site for the T allele variant is not (Wang et al. 2003a). Abnormalities in glycosylation have been associated with disease (Chui et al. 2001; Freeze and Westphal 2001; Nihlen et al. 2001; Scanlin and Glick 2001; Schenk et al. 2001). N-linked glycosylation at residue Asn129 possibly interferes with SP-B protein processing, secretion, and folding under certain disease conditions, resulting therefore in lower amounts of mature SP-B. Heterozygous SP-B (–/+) mice under normal conditions were identified with half of the SP-B amount found in homozygous SP-B (+/+) mice and with small physiological lung abnormalities (Clark et al. 1997). However, ablation of the SP-B gene causes lethal postnatal respiratory failure, which is associated with atelectasis (Clark et al. 1995). Of interest, published data (Guo et al. 2001; Lin et al. 2000) show the SP-B 1580_C allele as a risk factor in other pulmonary diseases (COPD and ARDS), and the T allele to confer protection from disease in the presence of a specific SP-A allele (Floros et al. 2001). The published data together with the results in this report indicate that the B1580_C variant may be a risk factor for several pulmonary diseases identified with derangement in normal lung function (Gregory et al. 1991; Gunther et al. 1999; Pison et al. 1995; Schmidt et al. 2002), which in turn may lead to alveolar collapse and permanent apposition of the denuded alveolar walls. These are common pathological findings in IPF and may play an important pathogenic role in the development of pulmonary fibrosis (Burkhardt 1989; Gunther et al. 1999; Honda et al. 2002; Myers and Katzenstein 1988; Schmidt et al. 2002). However, differences probably exist in the mechanisms through which the SP-B 1580_C variant contributes to IPF and to other pulmonary diseases.

SP-A1 and SP-A2 variants have also been found to be risk factors for IPF in the present study. Currently, there is no available information to indicate the way in which these variants contribute to IPF pathogenesis. SP-A plays multiple roles in surfactant function and structure, in host defense, and in other functions, and is increased in the BAL fluid of IPF patients (Phelps et al 2003). Therefore, different SP-A variants may contribute to disease pathogenesis under certain conditions in several ways. Our published studies have identified quantitative (Hoover and Floros 1999; Karinch et al. 1997; Wang et al. 2003b) and qualitative (Garcia-Verdugo et al. 2002; Wang et al. 2000, 2002) differences between SP-A1 and SP-A2 gene products and/or among SP-A alleles. For example, biochemical studies (Garcia-Verdugo et al. 2002) have shown differences between SP-A1 and SP-A2, and subtle differences between alleles. These include differences in structural stability, in self-aggregation, and in the ability of SP-A to induce aggregation of rough lipopolysaccharide (LPS) and of lipid vesicles. Of interest, SP-A1 allele differences in LPS aggregation have been observed, with the $6A^4$ allele showing an inhibition, perhaps attributable to amino acid differences in the CRD (Garcia-Verdugo et al. 2002). In the present study, by using a simplified system, we observed differences in self-aggregation between the $6A^{4t}$ and another truncated SP-A allele that differs from the $6A^{4t}$ allele only at amino acid 219 in the CRD region. Indeed, the SNP at amino acid 219 that approached significance (P=0.02) appeared to be a risk factor for IPF. The mechanism as to how the $6A^4$ allele may contribute to the pathogenesis of IPF is unknown. However, biochemical differences among SP-A alleles may be translated into differences in their ability to maintain the function, stability, and structure of surfactant.

An extreme example of this possibility is seen in pulmonary surfactant isolated from gene-targeted SP-A null mice. This surfactant is deficient in tubular myelin, a structural form of surfactant, and exhibits surface tension-lowering activity that is easily inhibited by serum proteins in vitro (Korfhagen et al. 1996). In this regard, SP-A biochemical differences in alleles may also be accentuated in the alveolar microenvironment of the IPF patient. For example, an imbalance between reactive oxygen species and available antioxidant defenses have been observed and thought to play a role in the pathogenesis of IPF (Beeh et al. 2002; Behr et al. 1995; Cantin et al. 1989; Lenz et al. 1996). In a microenvironment with increased oxidative stress, the SP-A may be differentially oxidized, and this may in turn have a differential impact on its structure and function. This possibility is consistent with our findings for the truncated alleles $1A^{0t}$ and $6A^{4t}$, in the model system used in the present study, following ozone-induced oxidation of the truncated alleles. The $6A^{4t}$ allele exhibited a higher level of self-aggregation following oxidation than did the $1A^{0t}$ allele. Furthermore, quantitative differences in SP-A mRNA among unrelated individuals (Floros et al. 1991; Karinch et al. 1997) or among alleles (Hoover and Floros 1999; Wang et al. 2003b) have been observed, and these may also, under certain conditions, be critical and contribute to the health or disease status of an individual.

In the context of the pathogenesis of IPF, it is important to emphasize that surfactant proteins may participate in extracellular matrix remodeling. SP-A has been shown to increase collagen expression by human lung fibroblasts (Vazquez de Lara et al. 2000) and can alter macrophage production of some matrix metalloproteinases (Vazquez de Lara et al. 2003). In addition, we recently found the SP-A levels to be elevated in the BAL and lung tissue of IPF patients (Phelps et al. 2003). Another important consideration for implicating SP-A in IPF is related to the role of SP-A as a natural inhibitor of type II pneumocyte apoptosis through interaction with a specific cell surface receptor (White et al. 2001). The integrity of the alveolar epithelium seems to be a critical determinant in the pathways that initiate fibrogenesis in the lung (Adamson et al. 1988; Selman et al. 2001). In this context, a number of observations in IPF lung specimens have revealed evidence of programmed cell death in alveolar epithelial cells. Epithelial apoptosis has been shown to occur in otherwise normal areas of the lung parenchyma or co-localizing with areas of fibroblastic/myoblastic foci (Barbas-Filho et al. 2001; Kuwano et al. 1996; Maeyama et al. 2001; Uhal et al. 1998). SP-A has also been shown to inhibit surfactant lipid-induced fibroblast apoptosis (Vazquez de Lara et al. 2000). Therefore, the anti-apoptotic effect of SP-A on either alveolar epithelial cells and/or fibroblasts may contribute to the development of IPF, and the anti-apoptotic capacity of different variants may change under certain microenvironmental cellular conditions.

In summary, our findings reveal: (1) significant associations with SP-A and SP-B SNPs and IPF but not with SP-C and SP-D SNPs; (2) the SP-B (B1580_C) and the SP-A ($6A^4$) alleles as risk factors for pulmonary disease; (3) the SNP at amino acid 219 of the $6A^4$ allele to be responsible for biochemical differences between $6A^4$ and other SP-A alleles. We speculate that surfactant protein SNPs that change the encoded amino acid result in subtle changes in surfactant protein structure and/or function. These differences under certain conditions may be accentuated and contribute to an increased susceptibility to the occurrence of disease via derangement in surfactant protein structure and/or function. Therefore, the surfactant protein variants may constitute part of an unknown number of susceptibility genes or gene modifiers or be linked to the susceptibility loci for IPF. Moreover, the present findings indicate that these marker loci may help identify individuals at risk for IPF. Further experimentation is warranted to confirm the associations made in this case-control study and to study mechanisms involved in IPF pathogenesis.

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