Differences in N-linked glycosylation between human surfactant protein-B variants of the C or T allele at the single-nucleotide polymorphism at position 1580: implications for disease

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Human surfactant protein-B (SP-B), a hydrophobic protein, is essential for normal lung function. SP-B is expressed and secreted by specific lung cell types, i.e. alveolar type II and Clara cells, of the respiratory epithelium. The SP-B precursor (42 kDa) undergoes post-translational processing to generate an 8 kDa mature SP-B. A single-nucleotide polymorphism (SNP) at nucleotide 1580 (C/T) in exon 4 of SP-B that changes amino acid 131 from threonine to isoleucine (Thr¹³¹ → Ile) is associated with several pulmonary diseases. The Thr¹³¹ → Ile substitution can eliminate a potential N-linked glycosylation site, Asn¹²⁹-Gln-Thr¹³¹, which is present in the SP-B variant of the C allele (ACT/Thr) but not in that of the T allele (ATT/Ile). To determine whether the C allele SP-B variant is indeed glycosylated at Asn¹²⁹-Gln-Thr¹³¹, we first generated stably transfected Chinese hamster ovary cell lines that expressed each version of SP-B, and developed specific SP-B polyclonal anti-peptide antibodies. Using both the stably

transfected cell lines and fetal lung explants, we observed that the C allele variant is indeed glycosylated at the Asn¹²⁹-Gln-Thr¹³¹ site, whereas the T allele variant, which served as a control, is not. In addition, we also confirmed that both SP-B variants contain another N-linked glycosylation site, Asn³¹¹-Ser-Ser³¹³. Given its association with several pulmonary diseases, this finding provides useful information for future studies in disease systems associated with this SNP. Further, we speculate that, given the fact that this SNP is found frequently in the general population, N-linked glycosylation at residue Asn¹²⁹ interferes with SP-B processing, secretion and folding under certain disease conditions.

Key words: CHO cells, fetal lung explants, protein modification, protein processing.

INTRODUCTION

Human surfactant protein-B (SP-B), a hydrophobic protein, is essential for normal lung function [1], and plays an important role in lowering surface tension at the terminal air space in the lung. The human SP-B gene is located on the p12–p11.2 region of chromosome 2 [2,3], and its genomic [4] and cDNA [5] sequences have been characterized. SP-B expression is restricted to specific cell types, i.e. alveolar type II cells and Clara cells in the respiratory epithelium [6,7]. The human SP-B precursor is a 381-amino-acid molecule consisting of signal peptide, N-terminal peptide, N-flanking peptide, mature SP-B region and C-terminal region. The SP-B precursor undergoes several post-translational cleavages, some of which are specific to the alveolar type II cell [8,9]. The mature SP-B peptide found in bronchoalveolar lavage material comprises amino acids 201–279 of the precursor and has a molecular mass of approx. 8 kDa. A number of SP-B gene mutations that cause fatal pulmonary disease, such as congenital alveolar proteinosis, have been identified [10–12], and SP-B-deficient mice exhibit pulmonary dysfunction and die shortly after birth [13].

In addition to mutations in the SP-B gene, a number of polymorphisms have been described [12,14,15]. The single-

nucleotide polymorphism (SNP) at nucleotide 1580 (C/T) in exon 4 changes amino acid residue 131 from threonine to isoleucine (Thr¹³¹ → Ile), and has been associated with several pulmonary diseases [16–18]. The amino acid substitution (Thr¹³¹ → Ile) can eliminate a potential N-linked glycosylation site, Asn¹²⁹-Gln-Thr¹³¹, present in the SP-B variant of the C allele (ACT = Thr), but absent from that of the T allele (ATT = Ile). However, it is not known whether this site is glycosylated, and thus whether the two variants differ in this regard. Because the presence of an N-linked glycosylation constitutes a significant co- and post-translational modification, and especially because this SNP has been associated with various pulmonary diseases [16–18], we considered it important to investigate whether this potential glycosylation site in the SP-B variant of the C allele is indeed glycosylated.

In the present study, we generated SP-B stably transfected mammalian Chinese hamster ovary (CHO) cell lines for each of the two SP-B C/T allele variants, and developed SP-B specific antibodies. Then, using the stably transfected cell lines as well as fetal lung explants, we studied whether N-linked glycosylation does occur at the SNP1580 site of the SP-B variant of the C allele, but not in that of the T allele. In addition, we confirmed the presence of another glycosylation

Abbreviations used: ARDS, acute respiratory distress syndrome; CHO, Chinese hamster ovary; Endo F, N-endoglycosidase F; GMEM, Glasgow's modified Eagle's medium; SNP, single-nucleotide polymorphism; SP-B, surfactant protein-B.

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site in the C-terminal fragment that is present in both the SP-B T and C allele variants.

MATERIALS AND METHODS

Cell lines and cell culture conditions

The mammalian CHO-K1 cell line (A.T.C.C., Manassas, VA, U.S.A.; cat. no. CCL 61) was used to express the human SP-B variants. Cell culture techniques and culture media have been described previously [19]. In brief, the cells were cultured in Glasgow's modified Eagle's medium (GMEM; Invitrogen, Carlsbad, CA, U.S.A.) containing 10% (v/v) fetal bovine serum at 37 °C in a 5% CO₂ atmosphere. Stably pEE14-SP-B-transfected cell lines were selected for their ability to grow in glutamine-free GMEM supplemented with 25 µM methionine sulfoximine and 10% (v/v) fetal bovine serum. Stable transfection of cell lines was further confirmed using genotyping and Western blot analysis.

Fetal lung explant culture

Human fetal lung was obtained from aborted fetuses (15–16 weeks' gestational age) under protocols approved by the Institutional Review Board for Human Research at the Pennsylvania State University College of Medicine. Fetal lung explants were cultured as previously described [8]. Fetal lung was chopped into 1–2 mm³ pieces and cultured in Waymouth's medium on a gentle rocking platform (approx. 3/min) in a 5% CO₂ incubator at 37 °C. After overnight culture (approx. 16 h), new medium containing hormones (10 nM dexamethasone, 0.1 mM 8-bromo-cAMP and 0.1 mM isobutylmethylxanthine) was added to the explant cultures. These were then maintained for 5 days with daily medium change, after which time the explant tissues were frozen until further analysis. Total tissue protein was extracted from the fetal tissues and used for Western blot analysis. In some experiments, although we attempted to determine the presence of SP-B in the conditioned medium from the explant culture using Western blot analysis, we were unable to detect any significant signal on the blots.

Preparation of SP-B expression constructs and generation of stably transfected cell lines

Human cDNAs for the C and T allele SNP1580 variants of SP-B were produced from human lung tissue by reverse transcription-PCR amplification techniques. Human lung tissue was genotyped as described previously [12]. Total RNA was isolated according using RNA-Bee™ (Tel-Test, Inc. Friendswood, TX, U.S.A.), and the first cDNA strand was synthesized using Moloney-murine-leukaemia virus reverse transcriptase (Invitrogen) with antisense primer GW1043. This was then used as template for PCR amplification with a pair of primers: GW1042, 5' GGGGAATTCAAGCTTGAGGTGCCATGGCTGAGTCA 3'; GW1043, 5' GGGGAATTCACCAGTGAAGTGACTCCCGAGG 3'. The SP-B cDNA fragments from PCR were purified, digested with *Eco*RI and *Hind*III, and cloned into the *Eco*RI/*Hind*III site of the pEE14 expression vector. Two constructs, pEE14-SP-B-T1580 and pEE14-SP-B-C1580, were generated and the sequences were confirmed by DNA sequencing. The two constructs differed by only one nucleotide, at the SP-B polymorphic C/T1580 site. Expression was driven by the human cytomegalovirus immediate-early enhancer/promoter region. The constructs pEE14-SP-B-T1580 and pEE14-SP-B-C1580 were transfected into mammalian CHO-K1 cells, and stably transfected cell lines were obtained after selection as described in [19].

Preparation of epitope-specific antibodies

Two peptide antibodies (NFPROX and NFLANK) were generated as described in [8]. SP-B peptides (NFPROX, Ser¹⁴⁵–Leu¹⁶⁰; NFLANK, Gln¹⁸⁶–Gln²⁰⁰) were prepared commercially (Genmed Synthesis, South San Francisco, CA, U.S.A.), conjugated to keyhole-limpet haemocyanin via an N-terminal cysteine residue, emulsified in Freund's complete adjuvant and injected subcutaneously into New Zealand white rabbits. Booster immunizations comprised peptide conjugated to keyhole-limpet haemocyanin emulsified in Freund's incomplete adjuvant. The serum was harvested after four immunizations. In addition, antibodies generated previously against the same two peptides were used for comparison studies [8]. The results were identical with the two sets of antibodies.

Carbohydrate removal by N-endoglycosidase F (Endo F)

Endo F (also known as peptide N-glycosidase F; New England BioLabs, Beverly, MA, U.S.A.) was used for removal of carbohydrate residues from the protein. Endo F is an amidase that can remove N-linked oligosaccharide from a glycoprotein. Specifically, this is achieved by the ability of Endo F to cleave between the innermost *N*-acetylglucosamine (GlcNAc) and the asparagine residue of glycoproteins that contain N-linked oligosaccharides. For stably transfected cell lines, conditioned medium from a 5-day culture was used for Western blot analysis before or after Endo F treatment. Approx. 10 µg of total protein in the medium (approx. 30 µl of conditioned medium) was loaded into one well of the gel. In addition, 30 µl of conditioned medium was treated with Endo F and then loaded on to the gel. Specifically, we incubated 30 µl of conditioned medium with 4 units of Endo F in G7 buffer (50 mM sodium phosphate, pH 7.5) at 37 °C for 2 h, and the reaction was stopped by the addition of loading dye buffer that included reducing agents. For the fetal lung explant tissues, we ground tissues on ice in PBS and subsequently sonicated them on ice for 30 s. Approx. 100 µg of total protein was incubated with 6 units of Endo F in G7 buffer at 37 °C for 2 h, the reaction was stopped by the addition of loading dye buffer, and the sample was subjected to gel electrophoresis.

Gel electrophoresis and Western blotting

SDS/PAGE analysis was conducted as described previously [19] using a 4–15% or 8–16% (w/v) polyacrylamide gradient gel run at 90 V for 2 h. SP-B was detected using rabbit antisera to SP-B peptides NFPROX and NFLANK at 1:5000 dilution. Blots were exposed to X-AR film following enhanced chemiluminescence detection. In some experiments, blots were stripped to remove antibody by incubation in 2% SDS, 0.06 M Tris/HCl (pH 7.0), and 0.72 M 2-mercaptoethanol for 30 min at 50 °C, and then reprobed with a second primary antibody [8].

Genotype analysis of stably transfected cell lines and fetal lung explants

Total DNA was extracted from transfected cultured cells or fetal lung tissues. Fetal lung tissues were genotyped as described previously [16]. For genotype analysis of transfected CHO cell clones containing human SP-B cDNA, primer pair 167/604 (primer 167, 5' ACAAGATGGCCAAGGAGGC 3'; primer 604, 5' GCATACAGATGCCGTTTGACTCA 3') was used for PCR amplification. PCR products were digested with restriction enzyme *Dde*I and separated on an 8% PAGE gel. The sizes of the fragments of SP-B C and T alleles were 154 bp and 177 bp respectively.

RESULTS AND DISCUSSION

Generation of stably transfected SP-B-expressing cell lines and confirmation of transfected genotypes

To generate cell lines stably expressing SP-B, we carried out two independent transfection experiments using plasmid pEE14-SP-B-T1580 or pEE14-SP-B-C1580. After selection for 4 weeks in glutamine-free GMEM containing 25 μ M methionine sulfoximine, we obtained 21 and 12 clones from the pEE14-SP-B-C1580 and pEE14-SP-B-T1580 transfections respectively. Only four of the 21 pEE14-SP-B-C1580-selected clones and two of the 12 pEE14-SP-B-T1580-selected clones expressed SP-B, as assessed by Western blot analysis. Two clones (C-1 and C-8) of the SP-B C allele and one clone (T-12) of the T allele expressed higher amounts of SP-B than the other clones, and these were used in subsequent experiments. To confirm the SP-B genotype of each transfected clone studied, we isolated the genomic DNA and genotyped each clone. The results showed that clones C-1 and C-8 contained the C allele sequence of SP-B and clone T-12 contained the T allele sequence (Figure 1).

Characteristics of SP-B expression and secretion by CHO cells

The SP-B precursor undergoes several post-translational cleavages to give rise to the mature SP-B found in broncho-alveolar lavage fluid. The early cleavages shown in Figure 2 are not cell specific, but the later ones are [8,9], and therefore cells other than alveolar epithelial type II cells can be used for studying the early processing steps. Our results indicate that CHO cells do indeed express the SP-B precursor and carry out the non-cell-specific processing steps, as well as secreting these SP-B molecules into the medium. The secreted SP-B products comprise proSP-B molecules plus the two cleaved peptides (Figure 3). Thus the stably transfected SP-B-expressing cells are appropriate for studying differences in N-linked glycosylation between the SP-B variants containing the C and T alleles, since the glycosylation event occurs early in SP-B processing, in a step that is not cell-specific.

Differences in N-linked glycosylation between human SP-B C and T alleles in stably transfected CHO cell lines

Based on amino acid sequence, both the C and T allele SP-B variants have an N-linked glycosylation site at the Asn³¹¹-Ser-Ser³¹³ position. In addition, the C allele has another potential

N-linked glycosylation site [5], i.e. Asn¹²⁹-Gln-Thr¹³¹. Figure 3 (upper panel) shows the *in vitro* expressed SP-B products from the three cell lines in the presence and absence of Endo F treatment. These SP-B products were probed with the antiserum NFPROX, which detects proSP-B and the N-terminal cleavage peptide (see Figure 2). In the absence of Endo F treatment, for the two cell lines expressing the SP-B C allele (C-1 and C-8), two bands were detected, with molecular masses of 45 kDa (proSP-B with two N-linked glycosylation sites) and 21 kDa (N-terminal peptide with one N-linked glycosylation site). The cell line expressing the SP-B T allele (T-12) also showed two bands, with molecular sizes of approx. 42 kDa (proSP-B with one N-linked glycosylation site) and 17 kDa (N-terminal peptide without N-linked glycosylation site). After Endo F treatment, the two bands for the C allele from both cell lines decreased in size, to approx. 39 kDa (proSP-B with no N-linked glycosylation site) and 17 kDa (N-terminal peptide without N-linked glycosylation site). For the T allele, as anticipated, only one band (42 kDa) decreased in size, to approx. 39 kDa; the other (17 kDa) did not change (Figure 3, upper panel). Identical results were obtained when the independently produced NFPROX antiserum was used (see the Materials and methods section).

Similarly, we carried out Western blot analysis with the NFLANK antiserum, which recognizes the proSP-B sequence from residues 186 to 200 and detects both proSP-B and the C-terminal cleavage peptide (see Figure 2). As shown in Figure 3 (lower panel), in the absence of Endo F treatment, two bands were detected for the SP-B C allele (in the C-1 and C-8 cell lines), with sizes of approx. 45 kDa (proSP-B with two N-linked glycosylation sites) and 25 kDa (C-terminal fragment with one N-linked glycosylation site). The T allele (T-12 cell line) also showed two bands, with sizes of approx. 42 kDa (proSP-B with one N-linked glycosylation site) and 25 kDa (N-terminal fragment with one N-linked glycosylation site). After Endo F treatment, both of the C allele variant clones and the T allele clone showed two bands with sizes of approx. 39 kDa and 22 kDa, indicating that both SP-B variants are glycosylated at the Asn³¹¹-Ser-Ser³¹³ site.

Based on these data (Figure 3), we conclude that the SP-B C allele contains two functional N-linked glycosylation sites at residues Asn¹²⁹ and Asn³¹¹, whereas the T allele has only one N-linked glycosylation site at Asn³¹¹.

Differences in N-linked glycosylation between SP-B C and T alleles variants in fetal lung explants

To determine whether Asn¹²⁹ is glycosylated *in vivo*, and thus confirm the results from the stably transfected CHO cell lines, two fetal lung explants from two individuals were obtained and cultured. The genotypes of these explants at SNP1580 were C/T and C/C, and they are shown in Figure 4 (upper panel; lanes 1 and 2 respectively). After a 5-day culture, total protein prepared from the explant tissue (this may include both intracellular and to a lesser degree extracellular SP-B protein present in the alveolar tissue spaces) was either treated or not with Endo F at 37 °C for 2 h and subjected to electrophoresis in an 8–16% (w/v) polyacrylamide gradient gel. The Western blot was probed with the epitope-specific antiserum NFPROX. The results shown in Figure 4 (lower panel) indicate that N-linked glycosylation at the SNP1580 position of the C allele SP-B variant does occur *in vivo*. In the fetal lung explant with the C/T genotype, three bands were identified before Endo F treatment, with molecular masses of approx. 17, 20 and 21 kDa. After Endo F treatment, one major band was present of approx. 17 kDa, which corresponds to the N-terminal peptide in the absence of N-linked glycosylation. In addition, a band of very low intensity of approx. 16 kDa was also

Figure 1 Genotype analysis of the human SP-B gene in transfected cell lines

Total DNA was extracted from three stably transfected cell lines, and was genotyped for the SP-B SNP1580 polymorphism using PCR-based restriction fragment length polymorphism methods. The C-1 and C-8 clones express the SP-B variant containing the C allele (lanes 1 and 2 respectively), and the T-12 clone expresses the SP-B variant containing the T allele (lane 3). Lanes 4 and 5 contain controls of plasmid pEE14 SP-B-T (SP-B cDNA of T allele) and pEE14 SP-B-C (SP-B cDNA of C allele) respectively.

Figure 2 Partial SP-B processing

The human SP-B gene encodes a 381-amino-acid precursor. The locations of sequences recognized by the two epitope-specific peptide antibodies NFPROX (Ser¹⁴⁶-Leu¹⁶⁰) and NFLANK (Gln¹⁸⁶-Gln²⁰⁰) are shown. Potential N-linked glycosylation sites are located at residues 311 (for both the C and T allele variants) and 129 (for the C allele variant only). The first 23 residues of the N-terminal sequence comprise the signal peptide. Mature SP-B is the region comprising residues 201-279. After removal of the signal peptide, proSP-B is cleaved at around residue 185. The C-terminal fragment is approx. 25 kDa with N-linked glycosylation, and the N-terminal fragment is approx. 17 kDa without N-linked glycosylation (T allele variant) or approx. 21 kDa with N-linked glycosylation (C allele variant). CHO cells lack the ability to process SP-B further; the subsequent processing steps are cell-specific.

Figure 3 Western blot analysis using epitope-specific peptide antibodies

The conditioned media from stably transfected cell cultures were treated or not with Endo F at 37 °C for 2 h, and subjected to electrophoresis in a 4-15% (w/v) polyacrylamide gradient gel. Upper panel: NFPROX antibody was used. This antibody recognizes the sequence Ser¹⁴⁵-Leu¹⁶⁰ of proSP-B, and detects proSP-B (42 kDa) and N-terminal cleavage peptides of 21 kDa (C allele variant) and 17 kDa (T allele variant) (see Figure 2). Lower panel: NFLANK antibody was used. This antibody recognizes the sequence Gln¹⁸⁶-Gln²⁰⁰ of proSP-B, and detects proSP-B (42 kDa) and the C-terminal cleavage peptide (21 kDa) (see Figure 2). Both panels show that cell lines C-1/C-8 and T-12 express the C and T allele variants respectively. The lane labelled CHO depicts the lack of SP-B protein in control untransfected CHO cells.

Figure 4 Genotype analysis of fetal lung tissues and Western blot analysis of human SP-B

Upper panel: fetal lung tissues were genotyped using PCR-based restriction fragment length polymorphism methods. Lanes 1 and 2 depict the genotype results for fetal tissue from individuals 1 and 2 respectively. Lanes 3-5 depict control genotypes for genomic DNAs from individuals homozygous for the C allele (lane 3), heterozygous for the C and T alleles (lane 4) and homozygous for the T allele (lane 5). Lower panel: Western blot analysis for detection of SP-B from 5-day-cultured fetal lung tissues. Total protein from fetal explant tissue, which may include both intracellular and secreted SP-B protein, was either treated or not with Endo F at 37 °C for 2 h and then subjected to electrophoresis in an 8-16% (w/v) polyacrylamide gradient gel. The Western blot was probed with the NFPROX antibody at a 1:5000 dilution. Following enhanced chemiluminescent detection, blots were exposed to X-AR film for 5 min (fetal lung tissue samples) or 30 s (cell lines).

visible. The fetal lung explant with the C/C genotype showed two bands, with molecular masses of 20 kDa and 21 kDa. These bands were decreased in size to 17 kDa (major) and 16 kDa (minor) after Endo F treatment. These results indicate that the N-terminal peptide of the SP-B C allele is indeed glycosylated

at the Asn¹²⁹-Gln-Thr¹³¹ site. It is not known why the N-terminal peptide of the C allele exists as two different sized bands (i.e. 20 and 21 kDa); this may be due to (a) alternative cleavage, (b) degradation after cleavage, or (c) different glycosylation. We speculate that the first two possibilities are more likely, because after Endo F treatment a band with very low intensity at approx. 16 kDa was present in samples with both the C/C and C/T genotypes. The intensity of the bands on the blot from the explants was lower than that with the stably transfected cell lines, probably because of the lower level of SP-B expression in the 16-week fetal lung explants (even after a 5-day differentiation in culture) compared with the transfected cell lines. The half-life of SP-B intermediates (N-terminal peptide) *in vivo* is not known.

Implications for disease

The alveolar lung epithelial type II cell is a polarized cell with both basal and apical surfaces [20–22]. Surfactant lipids and other surfactant proteins, including SP-B, are secreted in the form of lamellar bodies via the apical side of type II cells [23,24]. During SP-B processing, the N-terminal fragment that contains Asn¹²⁹ is cleaved in the endoplasmic reticulum [8]. Currently, the function (if any) of the N-terminal fragment of SP-B is not known. It is possible that N-linked glycosylation of this fragment plays a role in trafficking within the polarized type II cell [25,26]. Of possible interest and relevance are findings from published reports of genotype analysis. The SP-B T allele at the SNP 1580 (C/T) site, which lacks the N-linked glycosylation recognition site of the N-terminal fragment, is a protective factor against respiratory distress syndrome [17], whereas the C allele variant, which contains the N-linked glycosylation recognition site of the N-terminal fragment, is a risk factor for chronic obstructive pulmonary disease [18] and acute respiratory distress syndrome (ARDS) [16].

It is likely that N-linked glycosylation at Asn¹²⁹ does not interfere with the processing and secretion of SP-B under normal conditions, because the marker SNP1580 (C/T) is found frequently in the population [17]. However, the presence or absence of N-linked glycosylation may become important in certain disease states. For example, a higher level of SP-B-related immunoreactivity of approx. 25 kDa and 42–45 kDa was identified in serum from patients with ARDS [27]. Although the SP-B genotype at this SNP1580 (C/T) site was not determined in that study, it is of interest to note that in a different study of ARDS, the homozygous (C/C) genotype was found to be a risk factor for ARDS [16]. Whether the 25 kDa and/or 42–45 kDa SP-B immunoreactivity relates to the genotype at amino acid 131 remains to be determined. We speculate, based on the available data, that the presence or absence of the N-linked glycosylation site at Asn¹²⁹ in either the SP-B precursor molecule or the cleaved N-terminal fragment may play a role in several pulmonary diseases, although the detailed molecular mechanism(s) are currently unknown.

Conclusions

In summary, we have shown using stably transfected CHO cell lines, and confirmed using fetal lung explants, that the potential N-linked glycosylation site at the N-terminus of SP-B is indeed glycosylated. This differential N-linked glycosylation between SP-B C or T alleles at residue 129 may, under certain conditions, affect protein folding, transport through the endoplasmic reticulum to the Golgi and resistance to proteases [28,29]. Examples from the literature indicate that aberrant glycosylation of proteins

can lead to various human diseases [30,31]. However, in the case of the SP-B SNP1580 polymorphism, given its high frequency in the general population as well as its association with pulmonary diseases [16–18], it is likely that the involvement of these variants in disease pathogenesis becomes critical under specified (as yet unknown) conditions. Therefore the present findings provide useful information that indicates the need for further investigation into the differential N-linked glycosylation of SP-B in both health and disease.

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