Single nucleotide polymorphism in 5′-flanking region reduces transcription of surfactant protein B gene in H441 cells

Klaus H. Thomas, Philipp Meyn, and Norbert Suttorp

1Department of Internal Medicine/Infectious Diseases and Respiratory Medicine, Charité-Universitätsmedizin Berlin, Campus Mitte, Berlin; and 2Hospital Wangen, Wangen, Germany

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Thomas, Klaus H., Philipp Meyn, and Norbert Suttorp. Single nucleotide polymorphism in 5′-flanking region reduces transcription of surfactant protein B gene in H441 cells. Am J Physiol Lung Cell Mol Physiol 291: L386–L390, 2006. First published February 24, 2006; doi:10.1152/ajplung.00193.2005.—Surfactant protein (SP)-B is expressed in a cell-specific manner and is essential for surfactant function and survival. Abnormal surfactant function occurs in humans and genetically engineered mice with SP-B levels well below 50% of normal. SP-B mRNA levels vary in fetal lung explants among individuals, possibly due to genetic variety. Polymorphisms within the SP-B gene may affect mRNA content, but altered gene transcription or mRNA-stability has not been clearly demonstrated. We characterized a single nucleotide polymorphism (SNP) found in the upstream enhancer of SP-B, consisting of a single base pair change in the consensus sequence of the most downstream-located thyroid transcription factor 1 binding element in the upstream enhancer of the SP-B 5′-flanking region and located at position 384 upstream of the transcriptional start site of the SP-B gene. In a small patient population (n = 53) we found 70% were homozygous for the wild type (WT), one individual (2%) was homozygous for the polymorphism (Pm), and 28% were heterozygous. To further elucidate possible functions we performed electromobility shift assays with extracts from H441 cells that showed a reduced binding affinity of the mutated sequence compared with WT. In reporter gene assays the Pm caused a reduction of 53% in transcriptional activity compared with WT in transfected H441 cells. Stimulation of these constructs with retinoic acid resulted in enhanced reporter gene activity of both constructs. After stimulation the Pm still exhibited a reduced activity compared with the WT sequence. We conclude that the described SNP causes differences in SP-B transcriptional activity and thus may contribute to individually different SP-B mRNA levels.

Address for reprint requests and other correspondence: K. H. Thomas, Charité Universitätsmedizin Berlin, Dept. of Internal Medicine/Infectious Diseases and Respiratory Medicine, Campus Mitte, Schumannstrasse 20/21, 10117 Berlin, Germany (e-mail: klaus.thomas@charite.de).

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Fig. 1. Structure of the 5′-flanking region of surfactant protein (SP)-B. Numbers denote consensus binding sequence of the corresponding transcription factor. Sequences for the wild type (lower sequence) and the polymorphism (gray box) are also given. RARE, retinoic acid (RA) receptor element; TTF-1, thyroid transcription factor 1; HNF-3, hepatocyte nuclear factor 3.

METHODS

**DNA preparation.** A total of 200 μl of citrate blood was obtained from 53 patients of European descent. Samples were randomly taken from patients being hospitalized because of various medical conditions (80% pulmonary, 20% nonpulmonary). 28% chronic obstructive pulmonary disease, 13% bronchogenic carcinoma, and 10% pneumo-
tions (80% pulmonary, 20% nonpulmonary 28% chronic obstructive pulmonary disease, 13% bronchogenic carcinoma, and 10% pneumo-
nia; among those with <10% were interstitial lung diseases, asthma, pleural diseases, sleep apnea, and extrapulmonary malignancies). DNA from blood was prepared with a DNA extraction kit from Qiagen (Hilden, Germany). Informed consent was obtained before the procedure and confidentiality maintained according to the guidelines of and after approval by the Ethics Committee of the University of Marburg (Marburg, Germany). Patient blood samples received a specific number and were analyzed in an anonyized fashion.

**Converted PCR.** For genotyping, the 5′-flanking region of SP-B (nucleotides −980 to +39) was amplified with PCR using the following primers: sense 5′-AGC CCC ATC TCT TTT CAT CA-3′, antisense 5′-CAC TGC AGG AGG TGT GAC TC-3′. The amplified product was used for a nested PCR because direct amplification of a smaller product from genomic DNA was unsuccessful. The sense primer included a base pair mismatch at the 3′-end, creating a unique restriction site in the PCR-amplified product for XbaI in a mutation. The sequences of the primers for the nested reaction were sense 5′-GGA AGC TCT CAA GAG CAT TGC T-3′, antisense 5′-GAG GTA GGT GGA GGG CAT CT-3′.

**Cloning.** For cloning of the mutated and the wild-type (WT) sequence we used the amplified PCR product of the first PCR and performed a second nested PCR with primers containing specific restriction sites that allowed cloning into the pGL3 promoter vector (Promega, Mannheim, Germany). The primers were sense 5′-ATT TGC TAG CGG GAA AAG GTA AGG AGG-3′ containing a NheI site at the 5′-end and antisense 5′-AGC CTA GAT CTT CCC CTC CCA TC-3′ containing a BglII site at the 5′-end. The product represented the region from nucleotides −484 to −340 and thus included the entire enhancer region with all retinoic receptor-responsive elements and TTF-1 binding elements.

After restriction with the corresponding enzyme, products were cloned into NheI and BglII sites of the pGL3 promoter vector (Promega). The clone was propagated in XL-1 Blue (Stratagene, La Jolla, CA). Clone identity was checked by restriction digest and sequencing.

**Preparation of nuclear extract and EMSA.** Preparation of nuclear extract was previously described (8). H441 cells that showed 60–80% confluence were washed once in 1× PBS, scraped, and centrifuged for 3 min at 4°C. The cell pellet was resuspended in 500 μl of hypotonic buffer (mM: 10 HEPES, 1.5 MgCl2, 10 KCl, 0.5 DTT, and 0.2 PMSF, with 2 μg/ml aprotinin and 25 μg/ml leupeptin) and incubated on ice for 15 min. Cells were homogenized in a glass homogenizer with a type B pestle and vigorously vortexed for 30 s. After centrifugation for 5 min at 16 000 g, nuclei were resuspended in 100 μl of low-salt buffer (mM: 20 HEPES, 1.5 MgCl2, 0.02 KCl, 0.5 DTT, 0.2 PMSF, and 0.2 EDTA, with 2 μg/ml aprotinin, 25 μg/ml leupeptin, and 25% glycerol). An equal volume of high-salt buffer was added in a dropwise fashion under constant agitation. Nuclei were extracted for 30 min at 4°C and centrifuged for 20 min at 16 000 g. The supernatant was divided into aliquots and frozen in liquid nitrogen. Protein concentration was determined by Bio-Rad assay. Nuclei were stored at −85°C until use. Labeled oligonucleotides were purchased from Metabion ( Martinsried, Germany). The sequence of oligonucleotide containing the polymorphism (PM) was (consensus sequences of the PM/WT are underlined) sense 5′-CTC TCA AGA GCA TGG CTG AAA AGT AG-3′ and antisense 5′-CTA TTG AGC AAT AGT CCT CTG GAG AG-3′. The oligonucleotide containing the WT sequence was sense 5′-CTC TCA AGA GCA TGG CTG AAA AGT AG-3′ and antisense 5′-CTA TTG AGC AAT AGT CCT CTG GAG AG-3′. The following competitor oligonucleotides were used: specific sense 5′-CTC TCA AGA GCA TGG CTG AAA AGT AG-3′, nonspecific sense 5′-GCA TTG CTA GCC AGG-3′, antisense 5′-TCC ACT GGA TAG ACC TGC-3′. Labeled double-stranded probe (0.2 ng) was incubated with 10 μg of protein in 20 μl of 20 mM HEPES (pH 7.9), 70 mM KCl, 1 mM DTT, 1 mM EDTA, 12% glycerol and 1 μg of poly(dI-dC) at 37°C for 5 min. After incubation the DNA-protein complex was resolved on 5% nondenaturing poly-
acrylamide gel. The DNA-protein complexes were detected by a Odyssey infrared imaging system (LICOR).

**Cell culture and transfection.** H441 cells were purchased from the American Type Culture Collection (Manassas, VA) and maintained in RPMI medium supplemented with 2 mM glutamine, 10% fetal calf serum (PAA, Pasching, Austria), penicillin (100 U/ml), and amphotericin B (0.1 mg/ml) in a humidified atmosphere with 5% CO2. Cells were split, seeded in 60-mm dishes, and grown to ~50% confluence. We transfected each dish with 1 μg of plasmid with the luciferase reporter gene construct and a cytomegalovirus (CMV)-β-galactosidase (β-gal) plasmid (BD Biosciences Clontech, Heidelberg, Ger-
many), using Polyfect transfection reagent according to the instruction manual of the manufacturer (Qiagen, Hilden, Germany). Luciferase activity was measured after 2 days of incubation. Cell lysis and luciferase assays were performed with the Promega Bright-Glo luciferase assay system. Light units were assayed in a luminometer. For the experiments with all-trans RA, medium was replaced by fresh RPMI medium containing $10^{-5}$ M RA or control (DMSO) on the day after transfection. Luciferase activity was assayed after 2 days of incubation.

Statistics. Transfection efficiency was normalized to β-gal activity. Multiple transfections ($n = 5–12$) were performed, and results are presented as means ± SD. SD was <20% in all experiments, and statistical analysis was performed by two-tailed Mann-Whitney test. All results for transfection experiments were statistically significant with a $P < 0.01$.

RESULTS

Frequency. The polymorphism in the most downstream-located TTF-1 binding element of the enhancer region in the 5′-flanking sequence of SP-B consists of a SNP altering the core sequence from CAAG to CAAA. First, frequency of this polymorphism was analyzed with converted PCR that identified individuals carrying the mutation in the enhancer region of SP-B (Fig. 2) and allowed rapid screening. We found in 53 patients a frequency of 28% for WT/Pm heterozygotes ($n = 15$), 2% for Pm/Pm homozygotes ($n = 1$), and 70% for WT/WT homozygotes ($n = 37$).

In vitro binding properties of mutated TTF-1 consensus sequence. In EMSA, oligonucleotides comprising the second and third TTF-1 binding sites of the enhancer region (see Fig. 1) showed DNA-protein complexes with nuclear extracts from H441 cells. Cytoplasmic extracts did not cause any DNA-protein binding (results not shown). Only one complex appeared to be specific for TTF-1 binding because this DNA-protein complex was almost completely absent when specific nonlabeled competitor DNA in a 100-molar excess was added. In contrast, unlabeled nonspecific competitor DNA caused only a minor reduction of DNA-protein complex formation. Faint bands with higher and lower mobility were detected but did not show specificity. The oligonucleotide carrying the single mutation was associated with a decreased binding affinity compared with WT (Fig. 3). Although the decrease is moderate, we observed this effect constantly.

Functional significance of SNP. To assess the functional impact of the mutation we performed transfection assays with reporter gene constructs comprising the entire upstream enhancer region of SP-B with an SV promoter-driven luciferase reporter gene vector. The SV40 promoter in this setting has been shown to reliably reflect functions of the cis-acting elements in the 5′-flanking region of SP-B (17). We used a sequence spanning from nucleotide −484 to nucleotide −340 that has been shown to mediate most of the enhancer activity on SP-B transcription (13, 16, 17). Two days after transfection the mutation caused a reduction to 53% (±8.1) of reporter gene activity compared with WT (Fig. 4). Because RARE and TTF-1 are in close proximity and a strong interaction between the elements has been described (16), we investigated the response to $10^{-5}$ M all-trans RA. After exposure of transfected H441 cells to RA for 48 h both constructs increased reporter gene activity 1.6-fold (WT) and 1.7-fold (Pm), respectively (Fig. 4). These differences of stimulation by RA were not statistically significant ($P > 0.05$). However, the construct containing the mutated TTF-1 binding element still exhibited a reduced activity (64.7 ± 8.5%) after stimulation with RA compared with WT (Fig. 4).

DISCUSSION

We identified an SNP in the enhancer region of the 5′-flanking sequence of SP-B in a small population with a frequency of 28% for heterozygotes and 2% for homozygotes (frequency of the mutated allele 0.16, WT 0.84). These numbers are very similar to those that have been published by the Fred Hutchinson Cancer Research Center (http://pga.mbt.washington.edu/), which identified the mutated allele with a
frequency of 0.14 and the WT with a frequency of 0.86. Detection of homozygotes/heterozygotes was achieved by means of converted PCR and allowed a rapid screening. The SNP was located within a consensus binding sequence for TTF-1 and suggested a potential functional role in transcription of SP-B. In human fetal lung explants SP-B mRNA levels have been shown to vary among individuals (7, 9). This may be caused by an individually regulated transcription or mRNA stability. TTF-1 plays a pivotal role in the regulation and may carry a higher importance of “intermediate” enhancing transcriptional activity of reporter gene constructs (3rd and 4th bars). Values are means ± SD. *P < 0.01.

In summary, we characterized an SNP of a TTF-1 binding element in vitro and also showed a 50% reduction of reporter gene expression. Constructs containing the mutated or the WT sequence were stimulated to the same degree (~1.7-fold) by treatment with RA. However, the difference between the WT and the Pm after treatment with RA remained the same compared with nontreatment. Experiments by Naltner et al. (13) yielded slightly different results, with the WT being more susceptible to RA-stimulation than an arbitrarily mutated sequence that completely changed the TTF-1 core consensus sequence. One possible explanation is that in the study by Naltner et al. a construct that contained the entire 5′-flanking region with the SP-B promoter was used for transfection and RA stimulation experiments. Because the results for the isolated enhancer region and constructs containing the entire 5′-flanking region of SP-B correlated well for TTF-1 binding in a previous study (17), we focused on the isolated enhancer region. Another explanation is the possibility that the SNP may have a smaller impact on transcriptional activation after stimulation with RA. Because many cofactors and six binding elements in total (TTF-1 and RARE) are known to be involved in RA stimulation, there is a possibility that only a major change of the consensus sequence causes a significant reduction of activity of the transcriptional complex after RA stimulation, as shown by the study of Naltner et al. (13). In contrast, without RA stimulation the TTF-1 binding elements may carry a higher importance of “intermediate” enhancing activity of transcription.

TTF-1 belongs to the class of homeodomain proteins, and its binding activity is usually determined by contact between the homeodomain and the core DNA consensus sequence CAAG. Polymorphisms of TTF-1 binding regions show various alterations on transcriptional regulation. Fabbro et al. (6) investigated different mutations of the core and surrounding nucleotide sequences. Interestingly, they found a reduction of binding affinity of ~50% on a single base change from CAAG to CAAA (6). This resembles very closely the results we have found in the enhancer region of SP-B.

Despite the fact that about two-thirds of SP-B deficiency is caused by the homozygous 121ins2 mutation, Dunbar et al. (4) showed, particularly in heterozygotes for the 121ins2 mutation but also for other mutations, that a second additional variation can lead to absence of SP-B. Although the SNP we describe here most likely would not be sufficient alone to cause an apparent change of phenotype because SP-B levels that are known to cause lung disease usually are well below 50% of normal, it may still contribute to a loss of function caused by multiple mutations or to a predisposition for lung disease in combination with other environmental factors. Such environmental factors have been studied in an animal model of SP-B-knockout mice. Heterozygous SP-B (+/-) mice were more susceptible to hyperoxia- or LPS-induced lung injury than +/- mice (5, 14). In turn, overexpression of SP-B (+/++) inhibited endotoxin-induced lung inflammation (5). Whether combinatorial effects between the polymorphism described here and other polymorphisms or environmental factors occur in vivo is currently unknown.

In summary, we characterized an SNP of a TTF-1 binding element in the enhancer region of SP-B. The SNP caused a reduced binding of TTF-1 and diminished the transcriptional activity of the enhancer region of SP-B, and it may be a
contributing factor for reduced pulmonary SP-B levels and thus lung disease.

REFERENCES


