ABSTRACT

Most murine lung tumors are composed of differentiated epithelial cells. We have reported previously that surfactant protein (SP)-D is expressed in urethane-induced tumors. Serum levels of SP-D are increased in patients with interstitial lung disease and acute respiratory distress syndrome and in rats with acute lung injury but have not been measured in mice. In this study, we sought to determine whether SP-D could be detected in murine serum and discovered that it was increased in mice bearing lung tumors. Serum SP-D concentration was 5.0 ± 0.2 ng/ml in normal C57BL/6j mice, essentially absent in SP-D nulls, and 63.6 ± 9.0 ng/ml in SP-D-overexpressing mice. SP-D in serum was verified by immunoblotting. Serum SP-D was increased in mice bearing tumors induced by three different protocols, and the SP-D level correlated with tumor volume. However, in mice with a single adenoma or a few adenomas, SP-D levels were usually within the normal range. SP-D was expressed by the tumor cells, and there was also a field effect whereby type II cells near the tumor expressed more SP-D than type II cells in the remainder of the lung. Serum SP-D was also increased by lung inflammation. In airway inflammation induced by aerosolized ovalbumin in sensitized BALB/c mice, the serum levels were elevated after challenge. In conclusion, serum SP-D concentration is increased in mice bearing lung tumors and generally reflects the tumor burden but is also elevated during lung inflammation.

INTRODUCTION

One goal of cancer research is to identify a biomarker in serum that can be used to monitor tumor burden. Such a marker could be used to detect tumors in a relatively noninvasive manner and to evaluate the growth of tumors or their regression in response to therapy. For example, the value of the prostate-specific antigen test as a biomarker for prostate cancer and of β-human chorionic gonadotrophin for trophoblastic tumors is well recognized. For lung cancer, a variety of candidate serum biomarkers have been evaluated such as CA125 (11, 12). Although SP-D can be detected at very low levels in a variety of tissues, it is most highly expressed in type II cells and Clara cells (12, 13). SP-D is a collagenous glycoprotein whose monomeric structure is composed of an NH₂-terminal unit, a long collagenous portion, a neck region, and a highly conserved globular carbohydrate recognition domain that is the functional unit for attachment to microorganisms (11). SP-D binds to viruses, bacteria, yeast, and mycobacteria, and it can also alter inflammatory cell responses (11, 12). Mature SP-D is most commonly composed of four trimeric units with a molecular mass of about 516 kDa. SP-D is very similar to bovine conglutinin, a circulating protein (14). Although SP-D is conventionally referred to as a SP, it binds surfactant poorly and is not found in lamellar bodies. SP-D is probably not a true SP but part of the innate immune system.

In a preliminary survey of SP-D serum levels in inflammatory lung disease, we found an extremely high level in a BALB/c mouse that had a spontaneous adenocarcinoma. We therefore sought to determine whether serum SP-D could be used to detect lung epithelial tumors in mice.

MATERIALS AND METHODS

Animals. Unless stated differently, serum was collected from control mice by cardiac puncture in anesthetized mice. For each laboratory and tumor model, concomitant control samples were obtained so that genetic differences and variations in animal husbandry between control and the tumor-bearing mice were minimized. The genotype and phenotype of the SP-D transgenic and nulls were as reported previously (15, 16). For these experiments, all SP-D+/− mice were outbred into a NIH Swiss Black background eight times (15). SP-D transgenic mice express a rat SP-D cDNA driven by the human SP-C promoter (16). These SP-D transgenic mice express a 10–20-fold increase in SP-D in lavage fluid and lung homogenates (16). The SP-D transgenic mice were initially developed in C57BL/6 × C3H hybrids by pronuclear injection and subsequently outbred onto a NIH Swiss Black background eight times. Genotypes for mice subject to experimentation were confirmed by both PCR and DNA dot blot analysis.

Ovalbumin Sensitization and Challenge. BALB/c mice were sensitized and exposed to aerosolized ovalbumin to produce airway inflammation and hyperresponsiveness, as described previously (17, 18). In this protocol there are two control groups [i.e., one immunized by i.p. injection (IP only), and one that was not sensitized but received nebulized ovalbumin on three successive days (3 Neb only)]. The experimental group is sensitized by i.p. injection and subsequently outbred onto a NIH Swiss Black background eight times. Genotypes for mice subject to experimentation were confirmed by both PCR and DNA dot blot analysis.
hyperresponsiveness to methacholine, airway inflammation, and epithelial metaplasia (17). Serum samples were collected after the third day of nebulized ovalbumin or 24 h after the third nebulization. This experimental protocol was approved by the Institutional Animal Care and Use Committee of National Jewish Medical and Research Center.

**Urethane-induced Adenoma.** Male C57BL/6J mice at 6 weeks of age were purchased from Jackson Laboratory (Bar Harbor, ME). Breeder pairs from B6.A chromosome substitution strains (CSS or consomic strains; C57BL/6J mice bred to contain one A/J chromosome) were a gift from Dr. Joseph Nadeau (Case Western Reserve University, Cleveland, OH) and were bred to provide mice for experimental use at the University of Colorado Health Sciences Center for Laboratory Animal Care. Five A/J mice, 21 male B6.AY mice, and 7 male and 6 female B6.A14 CSS mice (each at 8 weeks old) all received i.p. injection with 1 mg/g urethane in saline as described previously (19). Twenty weeks after urethane treatment, mice were euthanized with a lethal dose of pentobarbital, and plasma was collected by cardiac puncture. Lung tumors were dissected and counted under a dissecting microscope (Wild Inc., Heerbrugg, Switzerland). Plasma was also collected from four age-matched A/J control mice (26 weeks of age).

**TGF-β Deletion and K-ras Mutation.** C57BL/6Ncr mice heterozygous for deletion of one TGF-β1 allele (TGF-β1−/−; Ref. 20) were mated with C57BL/6J/SV mice heterozygous for latent mutation of one K-ras allele (K-rasα+/−; Ref. 21). This mating generated four genotypes (TGF-β1−/−/K-rasα+/−, TGF-β1−/−/K-rasα+/−, TGF-β1−/−/K-rasα+/−, and TGF-β1−/−/K-rasα−/−). Confirmation of the genotypes was performed on DNA isolated from the tails of each offspring by using PCR amplification. Specific TGF-β1 and K-ras oligonucleotide primers were designed to distinguish heterozygous from wild-type littermates (20, 21). All mice were maintained in a pathogen-free barrier facility under conditions of constant room temperature (22 ± 1°C) and on a 12-h light/12-h dark cycle. Water and rodent food were available ad libitum.

**Inducible Activated K-ras Mouse Model of Lung Cancer.** Mice that form lung tumors due to expression of a tetracycline-regulated K-ras oncoprotein were generated as described previously (22). Bittaranigonuc (CCSP-rTA/het-op-K-rasY53f6(22)) animals and littermate controls were bred on wild-type (FVB), p53-deficient (FVB), or INK4a-Arf-deficient (C57BL/6) backgrounds. Genotyping was performed as described. The expression of activated K-ras was induced in lung epithelia by feeding adult animals (4–6 weeks of age) doxycycline-impregnated food pellets (Harlan-Teklad, Madison, WI) for varying amounts of time, ranging from 3 weeks to 11 months. Serum was collected by cardiac puncture, and the lungs were removed quickly. Total tumor number was determined, and the sizes of individual tumors were measured with a metric caliper. At all times, mice were treated in accordance with the guidelines for animal care and use established by the NIH.

**ELISA for Mouse SP-D.** Serum SP-D levels were measured by ELISA. Initially, the samples were coded, and the ELISA was performed without knowledge of the tumor status of the mice. When it was clear that some of the values for tumor-bearing mice were very high, appropriate dilutions of the serum were made based on the presence or absence of a tumor so as to avoid wasting serum. Recombinant mouse SP-D produced in Chinese hamster ovary cells was used as the SP-D standard. Polyclonal antirecombinant mouse SP-D rabbit IgG (10 μg/ml in 0.1 M sodium bicarbonate) was bound to wells in microtiter plates (Immuno 1 plates; Dynatech Laboratories, Alexandria, VA) overnight at room temperature. The wells were then incubated for at least 30 min at room temperature with a 4% (w/v) solution of nonfat dry milk in PBS containing 1% Triton X-100 (blocking buffer) to block nonspecific binding. The wells were washed with blocking buffer, and 100 μl of purified mouse SP-D (0–20 ng/ml) for standards or appropriately diluted mouse serum samples were added to each well. Plates were incubated for 90 min at 37°C and then washed with blocking buffer. One hundred μl of horseradish peroxidase-conjugated anti-SP-D antibody (10 μg/ml) were added to each well, and the plates were incubated for 90 min at 37°C. After washing with 1% Triton X-100 in PBS, 100 μl of the color-developing agent [0.1% O-phenylenediamine and 0.015% hydrogen peroxide in 0.1 M citrate buffer (pH 4.6)] were added. The reaction was carried out for 5 min at room temperature in a darkened room and was stopped by the addition of 100 μl of 2N sulfuric acid. Absorbance was measured with Microplate Autoreader EL-311s (Bio-Tek Instruments, Inc., Winooski, VT).

**SP-D Protein Identification.** SP-D was partially purified from serum, as described previously (9). SP-D binds Saccharomyces cerevisiae in a calcium-dependent manner. Aliquots of serum from normal, tumor-bearing, and SP-D/TNF-α transgenic mice were dialyzed against 5 mM TBS (pH 7.4) containing 1 mM EDTA to remove glucose, a competitive inhibitor. After dialysis, the sera were adjusted to 5 mM CaCl2 and incubated with paraformaldehyde-fixed S. cerevisiae cells (strain SEY6210) for 2 h at room temperature by gentle shaking. After incubation, the cells were washed three times with TBS containing 5 mM CaCl2. The cells were then incubated with 50 mM isositol for 1 h at room temperature to remove bound SP-D. The cells were centrifuged, and the supernatant was removed, pooled, and analyzed for SP-D by immunoblotting. The samples were placed in Laemmli reducing sample buffer, boiled, and layered onto precast 8–16% Tris-glycine polyacrylamide slab gels (Novex), and proteins were separated by electrophoresis in a Novex X cell Mini-cell (Invitrogen, Carlsbad, CA). Proteins were transferred to nitrocellulose membranes using the Novex X cell blot module according to the manufacturer’s instructions. Nonspecific binding sites on the nitrocellulose membranes were blocked by incubating the blots in 5% nonfat dry milk in 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, and 0.05% Tween 20 (TBBS; all from Sigma, St. Louis, MO) at 4°C overnight. The blots were incubated with rabbit antiserum SP-D IgG in 5% nonfat milk in TTBS (final concentration, 0.35 μg/ml) for 1 h at room temperature. Horseradish peroxidase-conjugated secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA) were added for 30 min at room temperature, and the antigens were detected by enhanced chemiluminescence (ECL-plus; Amersham Pharmacia Biotech, Piscataway, NJ) on Hyperfilm (Amersham Pharmacia Biotech).

**Immunostaining.** This was done with polyclonal rabbit IgG antimurine SP-D as detailed previously (6, 23).

**In situ Hybridization.** Tissues were fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned. The sections were dewaxed, hybridized with sense or antisense riboprobes to rat SP-D or SP-C, and processed as reported previously (6, 24, 25).

**Data Analyses.** All statistical tests were done with the SAS statistical analysis package (version 8.2). Significance level was set to 0.05 for all comparisons. For Table 1 and Fig. 1, we compared multiple groups with a single control group. Because the groups had different variances, Dunnett’s multiple comparison test was used with the Proc Mixed procedure where each group has its own variance. The nonparametric Wilcoxon’s rank-sum test was used for the comparisons in Fig. 2, A and B, and Fig. 5. For Fig. 4, both tumor volume and SP-D levels were first transformed to their logarithmic scale of base 10 because both variables were not normally distributed. The transformed data were then fitted to a linear regression equation using Proc GLM procedure. For the sensitivity and specificity analysis, tumor data were combined with normal control data. Because tumor volumes for normal group were all zero, logarithm (base 10) transformation was performed on (tumor volume + 1). A linear regression equation was derived to predict either tumor volume or SP-D if only one of them is known. We used whether or not the

Table 1. Serum SP-D concentration in control mice

<table>
<thead>
<tr>
<th>Animals</th>
<th>Strain</th>
<th>No. of animals</th>
<th>SP-D (ng/ml) (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6</td>
<td>84</td>
<td>4.8 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>FVB</td>
<td>12</td>
<td>5.0 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>A/J</td>
<td>9</td>
<td>8.3 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>Swiss Webster</td>
<td>5</td>
<td>4.9 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>BALB/c</td>
<td>4</td>
<td>4.2 ± 1.5</td>
<td></td>
</tr>
<tr>
<td>SP-D transgenic</td>
<td>Swiss Webster</td>
<td>3</td>
<td>63.6 ± 9.0</td>
</tr>
<tr>
<td>SP-D null</td>
<td>Swiss Webster</td>
<td>5</td>
<td>0.04 ± 0.03</td>
</tr>
</tbody>
</table>

a The C57BL/6 mice are derived from Jackson Laboratories stock at National Jewish Medical and Research Center as well as C57BL/6 from the National Cancer Institute.
b Plasma instead of serum was measured in the A/J mice. The ELISA was performed as stated in “Materials and Methods.”
c Groups that differ from normal control mice [all strains (P < 0.05)].
nulls.

As shown in Table 1, ovalbumin.4 Serum SP-D levels increased after challenge (Fig. 1). Allergen challenge in sensitized mice (26) and in mice challenged with nebulized ovalbumin in a well-characterized model of airway inflammation and hyperresponsiveness (17). We knew from previous work – were lower than values determined previously in rats (40 ng/ml).

Materials and Methods.

RESULTS

Normal Animals. Serum SP-D levels in normal mice (4–8 ng/ml) were lower than values determined previously in rats (40–80 ng/ml) and humans [70–110 ng/ml (7, 9)]. The serum level did not vary significantly with age, gender, or strain of mice. As shown in Table 1, SP-D was increased in transgenic mice that overexpress SP-D in type II cells driven by the SP-C promoter and was essentially zero in SP-D nulls.

Ovalbumin Sensitization and Challenge. In a preliminary study, we measured SP-D in sera of mice sensitized and challenged with nebulized ovalbumin in a well-characterized model of airway inflammation and hyperresponsiveness (17). We knew from previous work that lavage levels of SP-D were elevated after Aspergillus fumigatus allergen challenge in sensitized mice (26) and in mice challenged with ovalbumin.4 Serum SP-D levels increased after challenge (Fig. 1).

However, one value was excluded as an outlier because the value was 1420 ng/ml. This animal had a large tumor, which stained positive for SP-D (data not shown), and provided the rationale for the subsequent experiments.

Urethane-induced Adenoma. Because urethane-induced lung adenomas express SP-D (6), we evaluated serum from mice that had few [1–2 adenoma(s); CSS mice] or many (30–50 adenomas; A/J) adenomas. Serum SP-D was elevated in A/J mice bearing multiple tumors over their age-matched controls (Fig. 2A). All CSS mice received injection with urethane, and SP-D plasma levels were grouped according to whether the mouse did or did not develop lung tumors. Most CSS mice with 1 or 2 tumors had serum SP-D levels within the normal range, similar to that in animals bearing no tumors (Fig. 2B).

Serum SP-D levels in mice without tumors were slightly higher than those in other cohorts. This could be because all of these animals were exposed to urethane, these CSS strains might exhibit higher basal SP-D concentrations (basal levels have not been measured in these strains), or they may contain microscopic adenomas undetectable under the dissecting microscope.

We had reported previously that urethane-induced tumors expressed SP-D based on immunostaining (6). Here, we used in situ hybridization to demonstrate the relative mRNA levels in the tumors. Although these tumors expressed SP-D as expected (Fig. 3), there was also enhanced expression of SP-D mRNA in the type II cells surrounding the tumor. This proximity or field effect was not observed for SP-C expression. The implication is that the tumors produce a substance(s) that increases the expression of SP-D in neighboring normal type II cells.

Tumors Induced by Heterozygous Deletion of TGF-β and Activated K-ras. The next goal was to determine whether the rise in serum SP-D correlated with tumor volume. For this study, we chose a different mode of carcinogenesis. Heterozygous TGF-β1+/− mice (C57BL/6NCr TGF-β1+/−) were mated with K-ras+/− latent activating mice (C57 BL/6/129/sv K-ras+/−) to produce four genotypes. Wild-type (TGF-β1+/−/K-ras+/−) mice produced no tumors. Heterozygotes produce an intermediate number of tumors, and double heterozygotes (TGF-β1+/−/K-ras−/−) produced many tumors. The mice were sacrificed at 2–14 months of age, and the tumor diameter and number were recorded, and serum was collected. All tumors were assumed to be spheres for calculating tumor volumes. The remarkable observation was that the serum SP-D levels correlated with tumor volume on the logarithmic scale with an R2 value of 0.84 (Fig. 4). If one uses tumor volume as the response variable and SP-D as the predictor, the linear equation is Log10 (mm3 volume) − 2 value of 0.656 + 1.003 × Log10 (ng/ml SP-D).

Fig. 1. Serum SP-D levels in mice after ovalbumin sensitization and challenge.

Fig. 2. Serum SP-D levels in mice with urethane-induced adenoma. Because urethane-induced lung adenomas express SP-D (6), we evaluated serum from mice that had many adenomas (30–50 adenomas; A/J mice; A) and mice that had few adenomas (1–2 adenomas; CSS mice; B). Serum SP-D was elevated in A/J mice bearing multiple tumors over their age-matched controls. Shown in A are the results from four normal mice (3.8 ± 0.2 ng/ml) and five tumor-bearing mice (50.8 ± 6.3 ng/ml; P < 0.05). All CSS mice received injection with urethane, and the SP-D plasma levels were grouped according to whether the mouse did or did not develop lung tumors. Most CSS mice had only 1 or 2 tumors (n = 14) and had serum SP-D levels within the normal range, similar to that in animals bearing no tumors (n = 20; B). Serum SP-D levels in mice without tumors were slightly higher than those in other cohorts.

K. Takeda, unpublished observations.
serum values were greater than 11.4 ng/ml all had tumors in this data set. All samples from tumor-free mice had serum values of <11.4 ng/ml (specificity is 100%), and 80% of the tumor-bearing mice had serum values of >11.4 ng/ml (sensitivity is 80%). A serum level of 11.4 ng/ml corresponds to a total tumor burden of 1.84 mm³.

**Conditional Induction of Lung Tumors with Activated K-ras.**
To extend these observations to a third experimental system, we measured SP-D levels in serum from mice bearing lung adenomas and adenocarcinomas due to the induced expression of an activated K-ras (22). This protocol used bitransgenic animals (on wild-type or tumor suppressor-deficient backgrounds) in which the expression of murine K-ras4bG12D in lung epithelial cells is under the control of doxycycline. Tumors express SP-C, a type II cell marker, but not CC-10 (10-kDa Clara cell-associated protein), a Clara cell marker. This protocol was chosen because of the rapid induction of tumors and their propensity for malignant transformation. In our studies, mice on wild-type or tumor suppressor-deficient backgrounds were tested after the addition of doxycycline for 3 weeks to 11 months (22). Similar to the other inductive protocols, serum SP-D level differentiated the control mice from the tumor-bearing mice (Fig. 5).

**Verification of SP-D in Serum.** SP-D has been isolated from human and rat serum, and its identity has been verified by immunoblotting (8, 9). We isolated SP-D from serum by binding to yeast and eluting with inositol, as we reported previously (9). As shown in Fig. 6, a 43-kDa protein was isolated from serum, which has the same molecular mass as recombinant SP-D, and was recognized by a rabbit polyclonal IgG specific for murine SP-D. We chose serum from TNF-α-overexpressing mice as a positive control because we knew that the serum level in these mice was 343 ng/ml (n > 4). These mice have significant chronic lung inflammation (27). Under nonreducing conditions, both serum SP-D and recombinant SP-D showed higher-order oligomers characteristic of SP-D (data not shown).

**DISCUSSION**

Serum SP-D is elevated in mice with lung tumors, and the values correlate with tumor burden in this cross-sectional study. The ELISA measurement was reasonably sensitive and detected tumors greater than 1.84 μm³ (diameter, 1.52 μm). Serum SP-D was elevated in all types of tumors tested, which included chemically induced tumors, genetically induced tumors, and conditionally regulated mutant K-ras-induced tumors. However, an elevated serum SP-D is not specific for lung tumors and was also observed with lung inflammation. For example, serum SP-D was elevated in mice that overexpressed...
but, in general, is less responsive to alterations in culture conditions than expression of other SPs (36, 37). In vivo, pulmonary SP-D expression is increased by acute inflammation (endotoxin, antigen challenge, and so forth) and by overexpression of interleukin 4 (38, 39). We presume that some factor is expressed in the tumor that diffuses to the neighboring type II cells and increases SP-D expression. The factor could be produced by inflammatory cells within the tumors (40). However, this factor(s) has not been identified and will be the subject of future studies.

In summary, murine lung tumors express SP-D, and the tumor burden is reflected in the serum level of SP-D. However, serum SP-D is also elevated with lung inflammation, and SP-D is not specific for lung tumors.

ACKNOWLEDGMENTS

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Serum Levels of Surfactant Protein D Are Increased in Mice with Lung Tumors

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