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


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/6 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 tumor-associated antigen, carcinoembryonic antigen, and cytokeratin fragment 21.1 (CYFRA 21.1; Ref. 1–5). However, none has proven to be clinically useful, although high levels can indicate a large tumor burden.

In a recent study of urethane-induced adenomas in mice, we showed that these tumors expressed relatively high levels of SPs on the basis of immunostaining and in situ hybridization (6). These tumors all expressed SP-C, a marker restricted to alveolar type II epithelial cells, but not CC-10, a marker of nonciliated bronchiolar tumors all expressed SP-C, a marker restricted to alveolar type II cells. We have reported previously that surfactant protein (SP)-D is only SP-A and SP-D are hydrophilic and readily measured in serum. Serum levels of SP-A and SP-D are increased in acute lung injury and several interstitial lung diseases in humans (7, 8). In addition, serum SP-D is a biomarker of acute lung injury and inflammation in rats (9). SP-D also has the advantage that it should theoretically be useful in tumors derived from either type II cells or Clara cells. Wikenheiser and Whitsett (10), who studied murine tumors induced by SV40 early region genes under the control of the SP-C promoter, demonstrated that these murine tumors could express either CC-10, a marker of bronchiolar epithelial cells, or SP-C. SP-D was not measured in those mice.

SP-D is a calcium-binding lectin that is important in host defense (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 conglycinin, 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 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 to reprinted by permission. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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3 The important abbreviations used are: SP, surfactant protein; TGF, transforming growth factor; TBS, Tris-buffered saline.
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-C/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 Ca²⁺ with 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 (TTBS; all from Sigma, St. Louis, MO) at 4°C overnight. The blots were incubated with rabbit antiserum 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 antiserum 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

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**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>4</td>
<td>6.3 ± 0.90</td>
<td></td>
</tr>
<tr>
<td>SP-D null</td>
<td>5</td>
<td>0.04 ± 0.03*</td>
<td></td>
</tr>
</tbody>
</table>

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*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.

* Plasma instead of serum was measured in the A/J mouse. The ELISA was performed as stated in “Materials and Methods.”

* Groups that differ from normal control mice [all strains (P < 0.05)].
mouse had a tumor as the outcome variable and calculated the sensitivity and specificity for confidence limits of 95% or 99% for serum SP-D.

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−/− mice. 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 activation mice (C57 Bl/6/129sv 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 spherules 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 +1) = −0.656 + 1.003 × Log₁₀ (ng/ml SP-D). Only animals with tumors were included in this analysis to prevent anchoring the curve at the low end due to the large number of normal samples. Mice whose 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.

Fig. 1. Serum SP-D levels in mice after ovalbumin sensitization and challenge. BALB/c mice were divided into five groups. One group received two i.p. injections of ovalbumin (2 IP only; n = 8), a second group received nebulized ovalbumin on three successive days (3 Neb only; n = 6), a third group received i.p. sensitization and nebulized challenge and was sacrificed immediately after challenge (IPN 0 hr; n = 7), a fourth group was given the same treatment as the third group but sacrificed 24 hr after the third nebulized challenge (IPN 24 hr; n = 12), and a fifth group was sacrificed at 48 hr after challenge (IPN 48 hr; n = 12). Serum SP-D levels were measured by ELISA as stated in "Materials and Methods." *, P < 0.05.

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 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

* K. Takeda, unpublished observations.

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mouse had a tumor as the outcome variable and calculated the sensitivity and specificity for confidence limits of 95% or 99% for serum SP-D.

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−/− mice.

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

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4 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...
TNF-α, during respiratory syncytial virus infections, after ozone exposure, and after ovalbumin challenge in sensitized mice (some data not shown). Serum SP-D measurement is a relatively simple means of distinguishing normal mice from those with tumors or those with chronic inflammation. Serum SP-D will likely increase with tumor growth and decrease with regression, although this has not been tested in a prospective manner. Because measurement of tumor volume is difficult, and imaging techniques are not widely available, serum SP-D measurements should be useful to identify mice with tumors or to monitor response to therapy over time.

The importance of this finding for human lung cancer remains to be defined. Some human adenocarcinomas and large cell carcinomas express SP-D or SP-A, whereas small cell tumors and squamous cell tumors do not (28–32). However, about 75% of pulmonary adenocarcinomas express thyroid transcription factor 1, and about 50% of pulmonary adenocarcinomas express SP-A (32). Expression of SP-D should be similar to SP-A in adenocarcinoma (28). Although a large study of serum SP-D in human lung cancers has not been done, serum values of SP-D are elevated in some patients with adenocarcinoma and increase further if patients develop radiation pneumonitis (33, 34). To define the role of serum SP-D in human lung cancer, serum values of SP-D will need to be determined in a large cross-sectional study with multiple tumor histological types. In our murine tumor study, only mice with adenomas or adenocarcinomas were tested. Because normal human serum levels are higher and more variable than those in mice (7), serum SP-D levels may not be as distinctive a biomarker for lung tumors in humans. Serum SP-D levels may only be useful in that subset of patients with adenocarcinomas, such as the bronchioalveolar carcinoma subtype.

The mechanism responsible for the increased serum level of SP-D is not known. The most likely reason for this elevation is the production of SP-D by epithelial cells within the tumors. Tumor cells express SP-D mRNA and have detectable SP-D by immunostaining (6). Presumably, SP-D is secreted, enters the extracellular space, and reaches the systemic circulation via local lymphatic drainage. It is unlikely that the bronchoalveolar lavage fluid levels of SP-D would vary greatly depending on the presence or absence of tumors, although these values have not been measured to date. However, we cannot eliminate a contribution from the neighboring type II cells that overexpress SP-D. Although SP-D can be expressed on many mucosal surfaces, the highest concentration is found in the lung and bronchoalveolar lavage fluid (12, 13, 35). It is highly unlikely that the increased serum SP-D came from an extrapulmonary source. It is also unlikely that the volume of distribution or clearance of SP-D from the vascular compartment is altered in animals with tumors, although decreases in either parameter would theoretically increase serum concentrations.

The cause of the increased expression of SP-D in the type II cells surrounding the tumor is unknown. In vitro, SP-D expression is altered by factors that affect the state of differentiation of type II cells 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.

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