**Surfactant Protein-A Concentration in Sera from Dogs with Pulmonary Parenchymal Diseases**

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(Received 7 June 2012/Accepted 26 December 2012/Published online in J-STAGE 17 January 2013)

**ABSTRACT.** Pulmonary surfactant protein A (SP-A) is used as a biomarker to understand the clinical features of pulmonary diseases and associated prognostic indices in human medicine. This study was conducted to investigate whether or not serum SP-A concentration can be used as a biomarker for identifying pulmonary parenchymal diseases in dogs. Thirty-two dogs with pulmonary parenchymal diseases, 34 with nonrespiratory diseases and 57 healthy dogs were included. Serum SP-A concentration was measured in all dogs using sandwich enzyme linked immunosorbent assay with an anti-dog SP-A polyclonal antibody. Median serum SP-A concentration in healthy dogs was <2.0 ng/ml, whereas that in dogs with aspiration pneumonia (n=11), primary lung tumors (n=9) and blunt traumatic lung injury (BTLI; n=12) was 3.1, 7.2 and 2.6 ng/ml, respectively; these values were significantly higher than those in healthy dogs. The serum SP-A concentration in dogs with nonrespiratory diseases was comparable with that in healthy dogs. No correlation was observed between the serum SP-A and plasma C-reactive protein concentrations in dogs with aspiration pneumonia and BTLI. There was a significant correlation between the serum SP-A concentration and thoracic radiographic changes in dogs with BTLI. These findings suggest that the serum SP-A concentration may be a useful clinical biomarker of alveolar damage that can be used for differential diagnosis of pulmonary parenchymal diseases and nonrespiratory diseases in dogs.

**KEY WORDS:** antibody, diagnosis, respiratory, veterinary clinical pathology.

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Hypoxemia in small animals with pulmonary parenchymal diseases is usually caused by diffusion injury, shunt and ventilation/perfusion mismatch. In cases of dyspnea, the stress of an examination sometimes causes respiratory arrest, if it is not minimized [12]. There are many cases in which examinations under general anesthesia such as computed tomography, bronchoscopy, biopsy and bronchoalveolar lavage, which are difficult to perform, are required to establish a definitive diagnosis. Therefore, noninvasive examinations are required.

Pulmonary surfactant lines the alveolar surface and prevents collapse of small alveoli and enlargement of large alveoli by decreasing surface tension at the air–alveolar interface [27]. Surfactant is a mixture of lipid and protein [approximately 90% (w/w) lipids and approximately 10% (w/w) protein], and it plays an important role in gaseous exchange by increasing the lung surface area. Surfactant proteins comprise hydrophilic surfactant protein (SP)-A and SP-D and hydrophobic SP-B and SP-C; SP-A is generally the most dominant protein among these [5].

SP-A is a glycoprotein with a molecular weight of 28–36 kDa that belongs to the collectin family and is synthesized and secreted by alveolar type II cells and Clara cells [4]. Pulmonary collectins play an important role in innate immunity of the lung. SP-A acts similar to opsonin by binding directly and agglutinating with various pathogenic microbes, such as bacteria, viruses and certain fungi [1, 14, 29]. Furthermore, SP-A promotes bacterial phagocytosis by increasing cell surface expression of phagocytosis receptors (mannose receptor and scavenger receptor A) in macrophages and controls the inflammatory reaction of the pathogenic microbe component through interaction with Toll-like receptors and CD14 [10, 18].

SP-A concentration increases in the blood of humans with pulmonary diseases such as idiopathic pulmonary fibrosis [7, 11], pulmonary alveolar proteinosis [11], acute respiratory distress syndrome [2], cardiogenic pulmonary edema [2], bacterial pneumonia [19] and primary lung adenocarcinoma [28]. Therefore, blood SP-A concentration is a useful biomarker for understanding the clinical features of pulmonary diseases and associated prognostic indices [7, 19, 24–26]. In addition, SP-A can be used as a histopathological marker of pulmonary adenocarcinoma [17], and manifestations of SP-A are associated with lung cancer progresses [6].

Although the ideal method to measure serum SP-A concentration in dogs has not yet been established, we have recently developed an enzyme linked immunosorbent as-
say (ELISA) to measure the serum SP-A concentration in dogs for the first time [23]. The purpose of this study was to demonstrate that serum SP-A concentration is useful as a noninvasive, organ-specific blood biomarker for the differential diagnosis of respiratory diseases in dogs.

MATERIALS AND METHODS

Animals: Serum samples were obtained from 32 dogs with pulmonary parenchymal diseases, 34 with nonrespiratory diseases and 57 healthy dogs that were brought to Osaka Prefecture University and Okayama Animal Medical Center Hospital between March 2006 and July 2011. The dogs were in-hospital patients or outpatients and were owned by the animal hospital staff.

The pulmonary parenchymal diseases included aspiration pneumonia (n=11), primary lung tumors (n=9) and blunt traumatic lung injury (BTLI; n=12). The nonrespiratory diseases included pyometra (n=21), small intestinal obstruction (n=9) and pancreatitis (n=4) (Table 1).

Aspiration pneumonia was diagnosed on the basis of the following criteria: (i) witnessed or suspected regurgitation or vomiting episodes followed by acute onset of respiratory difficulty, cough or tachypnea; (ii) radiographic detection of pulmonary infiltrates in dogs at risk for aspiration; and (iii) identification of systemic disorders potentially associated with aspiration, including esophageal dysfunction (n=4), laryngeal disease (n=1), gastrointestinal tract disease (n=5) and decreased consciousness (n=1) [9].

Twelve dogs with BTLI were hit by cars, motorcycles or bicycles and thoracic injuries such as pulmonary contusion (n=12), pneumothorax (n=2) and rib fractures (n=2) were confirmed by thoracic radiography [15].

The primary lung tumors included adenocarcinomas (n=5), adenosquamous carcinomas (n=3) and histiocytic sarcoma (n=1) [3]. These tumors were removed by surgery, and pathological examination of the resected lungs was performed by veterinary pathology specialists at Osaka Prefecture University and Sumitomo Chemistry Techno Service Co., Ltd.

Dogs suffering from pyometra, small intestinal obstruction or pancreatitis were included to assess SP-A specificity. Pyometra was diagnosed on the basis of history, physical examination results, routine blood examinations, abdominal ultrasound findings and bacterial cultures of the contents of the resected uteri. Affected breeds included miniature Dachshunds (n=5); Golden Retrievers, Cavalier King Charles Spaniels and Pugs (n=2 each); Basset Hound, Shih Tzu, Chihuahua, Collie, Welsh Corgi and Shiba (n=1 each); and mongrels (n=4). Small intestinal obstructions were resected as foreign bodies by laparotomy. Affected breeds included miniature Dachshunds (n=4); Chihuahuas (n=2); Toy Poodle and Labrador Retriever (n=1 each); and mongrel (n=1). Pancreatitis was diagnosed on the basis of history; physical examination results; routine blood examinations, including canine pancreas-specific lipase (IDEXX Laboratories, Tokyo, Japan); and abdominal ultrasound findings. Affected breeds included English Cocker Spaniel, miniature Schnauzer, Papillon and mongrel (n=1 each).

Serum samples were not obtained specifically for this study, but were retrieved from blood samples that were not used for medical examinations and stored at −80°C. Therefore, the samples were collected at various times.

Thoracic radiographic severity score (TRSC): Nine thoracic radiographs of dogs with aspiration pneumonia, obtained on the day their blood was collected, were evaluated for the severity of pulmonary infiltrates using the method described by Kogan et al. [9]. The score was assigned on the basis of the severity (relative opacity) of interstitial and alveolar infiltrates. The scoring system used for the interstitial pattern was as follows: 1=mild, 2=moderate and 3=severe. The scoring system used for alveolar infiltrates was as follows: 4=mild, 5=moderate and 6=severe. The score was multiplied by the number of apparently affected lung regions (1–7) to provide a subjective radiographic severity score, with higher numbers reflecting more extensive pulmonary involvement (Fig. 1).
Radiographic evidence of pulmonary contusions was graded and assigned a score as follows: 0 = normal, 1 = mild, 2 = moderate or 3 = severe (Fig. 2).

**ELISA**: Serum SP-A concentration was measured by a method reported previously [23]. In brief, the polyclonal antibody against canine SP-A was obtained using purified canine SP-A from a rabbit. The antibody was further refined by protein G affinity chromatography, and the purified antibody [immunoglobulin G (IgG)] was biotinylated. These two antibodies were reacted with the normal dog serum (absorbed) and used in the ELISA.

Serum samples were diluted 1:10 in 0.67% bovine serum albumin (BSA) solution containing 3% Triton X-100 (BSAT). Purified SP-A was diluted with BSAT to create standard solutions. Sandwich ELISA was performed with microtiter ELISA plates. Wells were coated with antibody [50 µl; absorbed IgG solution of 5 µg/ml diluted in phosphate-buffered saline (PBS) solution] at 37°C for 1 hr.

Uncoated sites on the wells were blocked by incubation with 100 µl of 1% BSA solution at 37°C for 1 hr. A 50-µl aliquot of standard solution or serum sample was added to each well after washing the wells with PBS containing 0.1% TritonX-100, and the plates were incubated at 37°C for 1 hr. Biotin-labeled antibody (50 µl; diluted 1:1,000 in 0.67% BSA solution containing 0.1% Triton X-100) was added to each well after washing, and the plates were incubated at 37°C for 1 hr. The plates were then washed, 50 µl of horseradish peroxidase-conjugated streptavidin (Sigma Chemical Co., St. Louis, MO, U.S.A.) was added to each well after washing. The reaction was stopped after 15 min by adding 50 µl of 1 N HCl to each well. Absorbance was measured at 450 nm.

All samples were assayed in duplicate, and the standards were assayed in triplicate on each plate.

**Statistical analyses**: Data are expressed as median values (range). The serum SP-A detection limit obtained from the ELISA standard curve was 2.0 ng/ml, as reported previously [23]. The upper limit, defined as the peak value in which the mean ± two standard deviations of the serum concentration did not overlap, was 62.5 ng/ml. All the samples that were less than the detection limit were considered to be 1.9 ng/ml, and one sample that was beyond the upper limit was considered to be 62.6 ng/ml for statistical analysis.

The Mann–Whitney U-test was used for the statistical comparison between healthy dogs and dogs with either pulmonary parenchymal diseases or nonrespiratory diseases. Pearson’s correlation analysis was used to examine the relationships among variables (Statcel, the add-in forms on Excel, 3rd ed.; OMS Ltd., Tokyo, Japan). Statistical significance was defined as P<0.05.

**RESULTS**

**Healthy dogs**: Serum SP-A concentration in the healthy dogs was measured by ELISA (Fig. 3). Of the 57 healthy dogs, the serum SP-A concentration in 41 dogs was lower than the detection limit, and the median serum SP-A concentration was <2.0 ng/ml (range, <2.0–3.4).

**Aspiration pneumonia**: The median serum SP-A concen-
Serum SP-A concentration increases in various lung diseases in humans [2, 11, 19, 25, 28]. Lung diseases are categorized as interstitial and bronchoalveolar on the basis of the site of occurrence. The mechanism by which SP-A leaks from the alveoli into blood circulation is poorly understood. However, the cause of serum SP-A concentration elevation in patients with interstitial lung disease is thought to be, because of not only SP-A overproduction in the lungs but also leakage into the circulation by the injured basement membranes of the alveoli and vessels [18, 26]. Serum SP-A concentration is also elevated in human patients with bronchoalveolar lung disease, such as bacterial pneumonia [19]. Miyamoto reported that calves with experimentally induced bacterial pneumonia demonstrated proliferation of type II alveolar cells, increase in SP-A mRNA levels in the tissue and increase in serum SP-A concentration simultaneously on the third day, suggesting that increase in the serum SP-A concentration may be associated with bacterial pneumonia.

Table 2. Eleven dogs with aspiration pneumonia

<table>
<thead>
<tr>
<th>Breed</th>
<th>Timea) (days)</th>
<th>CRP (mg/dl)</th>
<th>SP-A (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cavalier King Charles Spaniel</td>
<td>0</td>
<td>0.9</td>
<td>5.3</td>
</tr>
<tr>
<td>Labrador Retriever</td>
<td>0</td>
<td>0.2</td>
<td>&lt;2.0</td>
</tr>
<tr>
<td>Mongrel</td>
<td>0</td>
<td>1.8</td>
<td>2.2</td>
</tr>
<tr>
<td>Yorkshire Terrier</td>
<td>0</td>
<td>6.9</td>
<td>3.3</td>
</tr>
<tr>
<td>Mongrel</td>
<td>1</td>
<td>19.0</td>
<td>10.1</td>
</tr>
<tr>
<td>Miniture Schnauzers</td>
<td>1</td>
<td>6.7</td>
<td>&lt;2.0</td>
</tr>
<tr>
<td>Shih Tzu</td>
<td>1</td>
<td>10</td>
<td>3.1</td>
</tr>
<tr>
<td>Yorkshire Terrier</td>
<td>1</td>
<td>12</td>
<td>&lt;2.0</td>
</tr>
<tr>
<td>French Bulldog</td>
<td>2</td>
<td>3.4</td>
<td>7.9</td>
</tr>
<tr>
<td>Miniture Schnauzers</td>
<td>2</td>
<td>12.0</td>
<td>2.1</td>
</tr>
<tr>
<td>Japanese Terrier</td>
<td>4</td>
<td>1.9</td>
<td>6.7</td>
</tr>
</tbody>
</table>

There was no significant correlation between the serum SP-A and plasma CRP concentrations (r=0.232, P=0.492). a) Elapsed time from the witnessed or suspected aspiration.

**DISCUSSION**

Serum SP-A concentration increases in various lung diseases in humans [2, 11, 19, 25, 28]. Lung diseases are categorized as interstitial and bronchoalveolar on the basis of the site of occurrence. The mechanism by which SP-A leaks from the alveoli into blood circulation is poorly understood. However, the cause of serum SP-A concentration elevation in patients with interstitial lung disease is thought to be, because of not only SP-A overproduction in the lungs but also leakage into the circulation by the injured basement membranes of the alveoli and vessels [18, 26]. Serum SP-A concentration is also elevated in human patient with bronchoalveolar lung disease, such as bacterial pneumonia [19]. Miyamoto reported that calves with experimentally induced bacterial pneumonia demonstrated proliferation of type II alveolar cells, increase in SP-A mRNA levels in the tissue and increase in serum SP-A concentration simultaneously on the third day, suggesting that increase in the serum SP-A concentration may be associated with bacterial pneumonia.
with proliferation of alveolar cells and leakage of SP-A into the blood stream, similar to that in interstitial lung disease [16]. Aspiration pneumonia is also a bronchoalveolar disease that results from inhaling oropharyngeal or gastrointestinal contents into the respiratory tract, triggering chemical, bacteriologic and immunologic damage to the airways [9]. The serum SP-A concentration was higher in dogs with aspiration pneumonia than in healthy dogs in our study. The reason may be that SP-A in alveoli leaks only from injured tissue just after aspiration, but SP-A overproduction was observed in addition to leakage after the beginning of proliferation of type II alveolar cells described above, which appeared on the third day or later in calf. However, the time of reactive change in alveolar cells may differ between animal species.

Table 3. Twelve dogs with blunt traumatic lung injury

<table>
<thead>
<tr>
<th>Breed</th>
<th>Time&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CRP (mg/dl)</th>
<th>SP-A (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>French bulldog</td>
<td>&lt;0.5</td>
<td>0.3</td>
<td>4.2</td>
</tr>
<tr>
<td>Miniature dachshund</td>
<td>&lt;0.5</td>
<td>0.1</td>
<td>&lt;2.0</td>
</tr>
<tr>
<td>Mongrel</td>
<td>&lt;0.5</td>
<td>9.0</td>
<td>3.4</td>
</tr>
<tr>
<td>Mongrel</td>
<td>&lt;0.5</td>
<td>NT&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;2.0</td>
</tr>
<tr>
<td>Papillon</td>
<td>&lt;0.5</td>
<td>0.0</td>
<td>&lt;2.0</td>
</tr>
<tr>
<td>Shih tzu</td>
<td>&lt;0.5</td>
<td>1.0</td>
<td>2.5</td>
</tr>
<tr>
<td>Siberian husky</td>
<td>&lt;0.5</td>
<td>0.1</td>
<td>2.5</td>
</tr>
<tr>
<td>Mongrel</td>
<td>&lt;1</td>
<td>9.8</td>
<td>3.9</td>
</tr>
<tr>
<td>Flat-coated retriever</td>
<td>3d</td>
<td>11.0</td>
<td>2.6</td>
</tr>
<tr>
<td>Golden retriever</td>
<td>5d</td>
<td>NT</td>
<td>2.8</td>
</tr>
</tbody>
</table>

There was no correlation between the serum SP-A and plasma CRP concentrations ($r=0.445$, $P=0.197$). a) Time, time to examination. b) NT, not tested.

Serum SP-A concentration in nine dogs with primary lung tumors was significantly higher than that in healthy dogs, and it did not seem to have an association with the size of tumors (Table 4). Furthermore, five dogs with adenocarcinoma in this study had high serum SP-A concentration, of which three dogs had extremely high serum concentrations. Serum SP-A concentration increases in the blood of human patients with primary lung adenocarcinoma [28]. Immunohistochemical studies have reported that human tumor cells show positive SP-A staining in approximately half of primary lung adenocarcinomas, but negative staining for metastatic lung carcinoma [17]. The reason why serum SP-A concentration increased in dogs was probably because SP-A in the alveolar space leaked into the blood from the normal tissue destroyed by the carcinoma and overproduction from the carcinoma itself, as in human patients. Therefore, serum SP-A concentration and its correlation with the distribution of SP-A in lung tissue of dogs with primary and metastatic lung tumors should be investigated in the future.

CRP is a useful acute-phase marker in dogs and humans and is routinely utilized as an indicator of inflammation to evaluate therapy and to monitor disease progression and prognosis in human medicine, although it is not specific to...
the lung [8, 20]. No significant correlation was observed between serum SP-A and plasma CRP concentrations in dogs with aspiration pneumonia or BTLIs in our study. An acute-phase response is observed in the lung following lung infection or injury, and the synthesis of pulmonary collectins may increase, serving a protective role by modulating inflammation and enhancing host defense [24]. However, the collectins are not classified as acute-phase reactants, because of their prolonged response kinetics and low induction levels compared to those of CRP. This may be the reason why the two proteins were not correlated.

The correlation between radiographic severity of lung infiltrates and serum SP-A concentrations in dogs with aspiration pneumonia was not significant, but it was significant in dogs with BTLI in our study. The reason why a correlation was not observed in dogs with aspiration pneumonia may be the difference in pathophysiology between the two diseases. In other words, BTLI is a clinical condition of lung injury that results from just an external force, but in aspiration pneumonia, the degree of tissue injury and infiltrative shadow in thoracic radiographs are different depending on what the patient has aspirated. An association between the disease severity on thoracic radiography at presentation and the oxygen administration time or hospitalization time in dogs with pulmonary contusion has been reported [21]. Prognostication may be possible by measuring the SP-A levels in BTLI.

The median serum SP-A concentration in healthy dogs was 2.0 ng/ml, and the mean blood concentration in healthy human subjects is reported to be 24.6 ± 9.6 ng/ml [25]. Although the reason for this difference is unknown, it may be because of a difference in species, the measuring method, or differences in antibodies and standards used to measure SP-A [24].

Some limitations of this study should be mentioned. This study was retrospective and included only a limited number of dogs. Many breeds were enrolled, and several factors may affect serum SP-A concentrations in dogs, including age, sex and body weight. We found no relationship between serum SP-A concentration and age, sex or body weight in our healthy dog population (data not shown). Therefore, we do not believe that differences in age, sex or weight of healthy dogs and the other dogs affected the results that were obtained. However, a cohort study will be required to confirm our results.

REFERENCES


