Serum concentrations of α_2 -macroglobulin, α_1 -antitrypsin, and C-reactive protein in dogs with suspected acute pancreatitis

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Abstract: In canine acute pancreatitis (AP), inappropriate release and activation of zymogen proteases within the pancreas results in the consumption of serum antiproteases. The aim of this study was to examine whether the serum concentrations of α_2 -macroglobulin (A2MG), α_1 -antitrypsin (A1AT), and C-reactive protein (CRP) differ between dogs with AP and healthy dogs. Twenty healthy dogs and 20 dogs with AP were included in this study. Concentrations of A2MG, A1AT, and CRP were measured in the sera of healthy dogs and dogs diagnosed with AP. Serum A2MG and A1AT concentrations were significantly lower in dogs with AP than in healthy dogs, whereas the serum CRP concentration was significantly higher. In addition, the concentrations of A2MG and A1AT were significantly higher in AP survivors than in AP non-survivors, while the CRP concentration was significantly lower. However, in both AP survivors and non-survivors, the CRP concentrations showed a negative correlation with A2MG concentrations but not with A1AT. These findings indicate that serum antiproteases and CRP concentrations might be associated with the mortality rate of AP in dogs.

Keywords: C-reactive protein, pancreas, protease inhibitor, trypsin

Introduction

Acute pancreatitis (AP) in dogs is defined as acute inflammation and/or necrosis in the pancreatic or peripancreatic tissues [1]. Severe AP has a high mortality rate ranging from 27% to 58%, although some cases are mild and self-limiting without complications [1-3]. In AP, zymogen proteases are inappropriately released and prematurely activated within pancreatic acinar cells, resulting in autodigestion [4]. Initial pancreatic acinar cell injury induces the release of various components including oxygen-derived free radicals, activating pancreatic proteases and phospholipase A₂ [5]. These active pancreatic proteases are then released into the peritoneal cavity [6] as well as the circulation [1]. Circulating active proteases lead to a proinflammatory state associated with the activation of neutrophils and macrophages and release of cytokines, such as tumor necrosis factor- α , interleukin (IL)-1, and IL-6, which subsequently induce C-reactive protein (CRP) synthesis [1, 7, 8].

Protease inhibitors, which form an important defense mechanism against developing AP, are found in tissue fluid and pancreatic tissue as well as serum [9]. The α -globulin fraction of serum consists of main protease inhibitors: α_1 -antitrypsin (A1AT), α_2 -macroglobulin (A2MG), inter- α -inhibitor, and α_1 -antichymotrypsin [10]. Of these antiproteases, A2MG may play a key role [11]. A2MG, a broad-specificity protease inhibitor, is able to bind to and clear pancreatic proteases as well as proteolytic enzymes released by neutrophils during inflammation [12]. Intravenously (IV) administered A1AT-trypsin complexes carry the enzyme to A2MG before elimination, suggesting that A1AT serves at least partly as a carrier [13]. IV infused trypsin is rapidly inhibited by A2MG, which binds to the trypsin and forms stable complexes [14]. Therefore, the objective of this study was to examine whether or not serum A2MG, A1AT, and CRP concentrations differ between dogs with AP and healthy dogs, and if so, whether there are differences in the serum concentrations of

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Conflict of Interest

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Fig. 1. Flow diagram of the enrollment of cases in this study. AP, acute pancreatitis; CKD, chronic kidney disease; MMVD, myxomatous mitral valve disease; HAC, hyperadrenocorticism; DM, diabetes mellitus; IMHA, immune-mediated hemolytic anemia; PLE, protein-losing enteropathy.

A2MG, A1AT, and CRP between AP survivors and AP non-survivors. In addition, the correlation between serum CRP concentrations and serum A2MG and A1AT levels in AP survivors and AP non-survivors was assessed.

Materials and Methods

Animals and ethical issues

The study approved by the University Ethics Committee (approval No. CBNUA-705-14-01), and the informed consent was obtained from the owners. Figure 1 shows a flow diagram describing the analysis of cases included in this study. Forty-five dogs with newly diagnosed AP were included in this prospective, observational cohort study. Among these 45 dogs, twenty-five dogs with concurrent diseases, including chronic kidney disease ($n = 5$), myxomatous mitral valve disease ($n = 3$), hyperadrenocorticism ($n = 3$), diabetes mellitus ($n = 2$), immune-mediated hemolytic anemia ($n = 1$), or protein-losing enteropathy ($n = 1$), dogs with recurrent bouts of AP ($n = 7$) and dogs referred from other hospitals (diagnosed with AP and hospitalized for > 1 days) ($n = 3$) were excluded on the basis of clinical data [15]. Thus, twenty client-owned dogs newly diagnosed with AP were enrolled in this study. They had not been treated with any medications associated with AP for at least 1 week before a diagnosis of AP was made. Twenty healthy, client-owned dogs were included as a control (Table 1). The healthy dogs were recruited when the dogs presented for health examinations and were enrolled on the basis of a normal clinical data.

Dogs with AP were classified as survivors ($n = 13$) and non-survivors ($n = 7$) by the attending clinician. Dogs were designated as survivors if they showed obvious clinical improvement and were discharged from the hospital. Evidences of the treatment in the survivors included resolving abnormal findings related with AP and negative result of

Table 1. Characteristics of dogs with AP and healthy dogs

Characteristics	Dogs with AP (n = 20)	Healthy dogs (n = 20)
Sex		
Female	5	4
Neutered female	4	4
Male	4	3
Neutered male	7	9
Body weight (kg)	5.23 ± 2.74 ; 2.1–10.8	6.79 ± 2.96 ; 3.8–12.0
Age (yr)	8.70 ± 3.60 ; 3–14	7.50 ± 3.50 ; 2–15
Breed		
Miniature Schnauzer	4	5
Miniature Poodle	2	1
Maltese	2	1
Shih Tzu	2	2
Yorkshire Terrier	2	2
Pomeranian	2	2
Dachshund	1	0
Cocker Spaniel	1	0
Beagle	1	3
Pekinese	1	0
Mixed breed	2	4

Data are shown as mean $\pm$ SE with ranges.
AP, acute pancreatitis.

SNAP canine pancreatic lipase (cPL) test (IDEXX Reference Laboratories, Korea) [15]. Dogs were considered non-survivors if they died during hospitalization.

Diagnosis of AP

For a diagnosis of AP to be established [8, 16], the following 5 criteria had to be met: 1) a history of acute onset of signs compatible with AP such as anorexia, vomiting, weak-

ness, or diarrhea; 2) an initial physical examination that included dehydration, icterus, fever, or abdominal pain; 3) abdominal ultrasound (Alpha 5; Aloka, Japan) findings suggestive of pancreatitis, including hypo/hyperechoic lesions, or mixed patterns, were recognized in pancreata that were potentially enlarged and irregularly shaped [8]. Furthermore, changes typically recognized as being secondary to pancreatitis, such as hyperechoic mesentery, localized free abdominal fluid, thickened duodenal or gastric wall, duodenal spasm, irritated appearance of the adjacent intestine, and dilated common bile duct, were also considered to be ultrasonographic evidence of AP; 4) increased specific (Spec) cPL concentrations (IDEXX Reference Laboratories) [15]. In the Spec cPL assays, concentrations $>400 \mu\text{g/L}$ were considered to be consistent with AP [17].

Healthy dogs were judged to be healthy based on the examination criteria of the previous paper [15].

Treatment and grouping

Treatment was conducted as currently recommended at the time of the study [8, 16]. Aggressive fluid therapy was performed to combat the effects of disturbed pancreatic microcirculation that contributes to progression from mild to severe pancreatitis [18]. Supportive treatment consisted of low-molecular weight heparin, analgesics, antiemetics, broad-spectrum antibiotics, and H_2 receptor blockers [15]. In the case of assumption of pain, butorphanol tartrate (Butophan; Myungmoon Pharm, Korea; $0.2 \text{ mg/kg IV every 6 h}$) or buprenorphine (NORSPAN patch; LTS Lohmann Therapie-Systeme AG, Germany; $4 \mu\text{g/kg/h TD every 4 days}$) was administered [19]. Maropitant citrate (Cerenia; Pfizer, France), an antagonist of neurokinin 1 receptors, was administered ($1 \text{ mg/kg SC every 24 h}$) as an antiemetic, and famotidine (Gaster; Dong-A ST, Korea), a H_2 receptor antagonist, was also given ($0.5 \text{ mg/kg IV every 12 h}$) [15]. Even though there is little evidence of bacterial involvement in the initiation of AP, the dogs received broad-spectrum antibiotics when there was evidence of pyrexia and neutropenia with a left shift. Nil per os was maintained until vomiting ceased. A small amount of water was supplied when vomiting ceased even if the antiemetic was discontinued. If there was no vomiting after water intake, the dogs were fed small amounts of commercial low fat diet (Gastro-intestinal low fat; Royal Canin SAS, USA) on the following day.

Dogs with AP were classified into "survivor" or "non-survivor" groups according to the previous report [15]. Evidences of the AP resolution included ameliorating symptoms related with AP and negative result of SNAP cPL test [14].

Assays

All serum samples from dogs with AP and healthy dogs were stored at -80°C until use. Samples were analyzed in duplicate in a single run with commercially available canine-specific enzyme-linked immunosorbent assay (ELISA) kits for A2MG (Canine α_2 Macroglobulin ELISA kit; MyBio-

Source, USA), A1AT (Canine α_1 Antitrypsin ELISA kit; MyBioSource), and CRP (Canine CRP ELISA kit; Millipore, USA) according to the manufacturer's instructions. For A2MG, both intra- and inter-assay coefficients of variation (CVs) were less than 10% with 1.0 ng/mL of sensitivity. For A1AT, both intra- and inter-assay CVs were less than 15% with $10 \mu\text{g/mL}$ of sensitivity. For CRP, intra- and inter-assay CVs were $\pm 3.5\%$ and $\pm 2.7\%$, respectively, with a sensitivity of 3.6 ng/mL . Optical density was determined at 450 nm using an automated microplate reader (EPOCH; Bio Tek, USA).

Statistical analyses

Statistical analyses were carried out using a commercially available statistical software program (Prism 6.05 for Windows; Graph-Pad Software, USA). D'agostino-Pearson tests were performed to determine whether the data were normally distributed. Mann-Whitney U tests were used to compare differences between dogs with AP and healthy dogs, and between AP survivors and AP non-survivors. We used Spearman's rank correlation tests to examine the correlation between serum concentrations of CRP and A2MG, and between CRP and A1AT, in dogs with AP. The p -values were calculated for 2-tailed tests, and the 95% confidence intervals (CIs) for the differences between medians were determined. For all analyses, values of $p < 0.05$ were considered statistically significant. The data were expressed as medians with ranges.

Results

Patient characteristics

The AP dogs included 11 spayed females, 7 castrated males, and 2 intact males, while the control dogs included 8 spayed females, 7 castrated males, 3 intact males, and 2 intact females. The mean age (years) was not significantly different between the AP group (8.70 ± 3.60) and the healthy group (7.50 ± 3.50), or between survivors (8.10 ± 3.40), and non-survivors (9.30 ± 3.80) in the AP group. There was also no significant difference in body weight (kg) between the AP group (5.23 ± 2.74) and the healthy group (6.79 ± 2.96), or between survivors (4.58 ± 2.03) and non-survivors (5.89 ± 3.29) in the AP group. Breeds in the AP group were Miniature Schnauzer ($n = 5$), Miniature Poodle ($n = 2$), Maltese ($n = 2$), Shih Tzu ($n = 2$), Yorkshire Terrier ($n = 2$), Pomeranian ($n = 2$), Dachshund ($n = 1$), Cocker Spaniel ($n = 1$), Beagle ($n = 1$), Pekingese ($n = 1$), and Mixed breeds ($n = 2$). The control healthy dogs included Miniature Schnauzer ($n = 5$), Miniature Poodle ($n = 1$), Maltese ($n = 1$), Shih Tzu ($n = 2$), Yorkshire Terrie ($n = 2$), Pomeranian ($n = 2$), Beagle ($n = 3$), and Mixed breed ($n = 4$).

Serum concentrations of A2MG, A1AT, and CRP in dogs with AP and healthy dogs

The median serum concentrations of A2MG (95% CI = -0.01 , -0.02 ; $p < 0.001$) and A1AT (95% CI = -0.34 , -0.72 ; $p < 0.001$) were significantly lower in dogs with AP than in

Fig. 2. Serum concentrations of (A) A2MG, (B) A1AT, and (C) CRP in dogs with AP (n = 20) and healthy dogs (n = 20). The horizontal lines indicate the medians. A2MG, α_2 -macroglobulin; A1AT, α_1 -antitrypsin; CRP, C-reactive protein; AP, acute pancreatitis. * $p < 0.05$ (Mann-Whitney U test).

Fig. 3. Serum concentrations of (A) A2MG, (B) A1AT, and (C) CRP in AP survivors (n = 13) and non-survivors (n = 7). The horizontal lines indicate the medians. A2MG, α_2 -macroglobulin; A1AT, α_1 -antitrypsin; CRP, C-reactive protein; AP, acute pancreatitis. * $p < 0.05$ (Mann-Whitney U test).

healthy dogs (Fig. 2A and B), whereas those of CRP (95% CI = 35.35, 63.80; $p < 0.001$) were significantly higher in

dogs with AP than in healthy dogs (Fig. 2C).

Fig. 4. Correlations between the serum concentrations of CRP and in (A) survivors ($n = 13$) and (B) non-survivors ($n = 7$) in dogs with AP, and correlations between levels of CRP and A1AT in (C) survivors and (D) non-survivors (Spearman's rank correlation test). A2MG, α_2 -macroglobulin; A1AT, α_1 -antitrypsin; CRP, C-reactive protein; AP, acute pancreatitis.

Serum concentrations of A2MG, A1AT, and CRP in AP survivors and AP non-survivors

Compared with AP non-survivors, the median serum concentrations of A2MG and A1AT (95% CI = 0, 0.01; $p = 0.002$ and 0.79, 0.55; $p = 0.0043$, respectively) were significantly higher in AP survivors (Fig. 3A and B), whereas those of CRP (95% CI = -4.56, -18.42; $p = 0.0112$) were significantly lower (Fig. 3C).

Correlations between CRP and A2MG and between CRP and A1AT in dogs with AP

In AP survivors, the serum concentration of CRP showed a negative correlation ($r = -0.6923$; $p = 0.0087$) with the serum A2MG concentration (Fig. 4A). In AP non-survivors, there was also a negative correlation ($r = -0.9286$; $p = 0.0067$) between the serum concentration of CRP and that of A2MG (Fig. 4B). However, CRP showed no correlations with the serum A1AT concentration ($r = -0.3846$; $p = 0.1944$; Fig. 4C) in AP survivors and with A1AT ($r = 0.4286$; $p = 0.3536$; Fig. 4D) in AP non-survivors.

Discussion

Several studies evaluated the concentrations of α -macroglobulin in AP and observed low serum α -macroglobulin concentrations in dogs with spontaneous AP [20] or experimentally-induced AP [21], and in humans with AP [6, 22]. In agreement with these reports, our study revealed that the

serum concentrations of A2MG were downregulated in dogs with AP. This can be ascribed to the consumption of the A2MG during AP, as supported by studies in which 30% of plasma A2MG in patients with AP was detected in complex with proteases [23]. We also found that serum A2MG concentrations in AP non-survivors were lower than those of AP survivors. No correlations have been reported between serum α -macroglobulin concentrations and the severity of spontaneous AP in dogs [20]. However, serum concentrations of A2MG in humans with severe AP showed greater decreases compared to humans with mild AP during hospitalization [6,24]. In addition, a reduction in serum A2MG was reported previously to be correlated with outcome in human AP [25]. These findings suggest that low concentrations of serum A2MG in AP dogs may also result from consumption of A2MG and that low levels of serum A2MG are predictive of a severe disease course of AP.

In the present study, serum A1AT concentrations were downregulated in dogs with AP. Serum A1AT concentrations were also lower in AP non-survivors compared with AP survivors. These results were supported by studies in humans that demonstrated lower serum concentrations of A1AT and a delay in rapid rises in serum A1AT concentrations and trypsin inhibitory capacity in severe AP compared to mild AP, suggesting the failure of patients with a severely compromised immune system to mount an inflammatory response [22]. However, these findings are contradicted by another report in which circulating concentrations of A1AT in humans

with severe AP were higher than those in mild AP [24]. In addition, a subsequent clinical study found that serum concentrations of trypsin 2-A1AT complexes were higher in AP patients than normal controls, and suggested that trypsin 2-A1AT complexes reflect AP-induced release of active trypsin 2 into circulation [26]. This report also suggested that serum trypsin 2-A1AT complex concentrations were more useful in predicting a severe course of disease at presentation than trypsinogen 2 and amylase concentrations [26]. Even though the serum concentrations of trypsin-antitrypsin complexes were not measured in this study, the finding of lower serum concentrations of A1AT in AP dogs compared to healthy dogs, and AP non-survivors compared to AP survivors, might be indicative of the active formation of trypsin 2-A1AT complexes and subsequent consumption of A1AT in AP dogs, which would be expected to be more excessive in dogs with severe AP and poor prognosis.

In human and veterinary medicine, it was proposed that fatal systemic pancreatitis arises from excessive leukocyte activation with the release of secondary mediators such as proinflammatory cytokines [1, 27]. CRP, the acute phase reactant, is synthesized by the liver in response to inflammation, infection, or tissue destruction. The serum concentration of CRP was reported to be increased in human subjects with AP [11] as well as in dogs with other inflammatory and infectious diseases, including experimentally-induced [28, 29] and spontaneous AP [2]. This is consistent with our results that serum concentrations of CRP were upregulated in dogs with AP. In the present study, serum CRP concentrations were also higher in AP non-survivors compared with survivors. In human medicine, Serum concentration of CRP was reported to be correlated with the severity of disease [9,30] and organ dysfunction in patients with AP [31]. This association is not restricted to AP, as elevated concentrations of serum CRP upon intensive care unit admission were correlated with an increased risk of organ failure and death [32]. Therefore, CRP is considered the gold standard serum marker for predicting severity of AP in humans [9]. Although CRP has not been evaluated with respect to severity of disease in AP dogs, our finding of high concentrations of serum CRP in AP non-survivors shows that, as in humans, serum concentrations of CRP might be useful for monitoring clinical progression and response to therapy in dogs with AP [10].

Our study showed that serum concentrations of CRP and A2MG were inversely correlated, whereas those of CRP and A1AT were not. Synthesis of CRP by the liver is largely regulated by IL-6 [33], and administration of recombinant human IL-6 in rats led to increased hepatic A2MG messenger RNA levels [34]. Conversely, CRP induces the rapid releases of cytokines including IL-6 and IL-1 [35], which accelerates the secretion of A2MG [19, 36, 37]. IL-1 β and IL-6 were reported to be high in AP dogs compared with healthy dogs [8]. Thus, correlations between serum levels of A2MG and CRP might be explained by changes in the serum levels of cytokines, including IL-1 or IL-6. Further examination of the associa-

tion between cytokines (IL-1, IL-6, and IL-10) and the production of A2MG and CRP is required to determine whether cytokines influence the balance between A2MG and CRP in dogs with AP.

This study had several limitations. One was that a small number of dogs with AP were enrolled in this study, which can produce false-positive results or allow over-estimation of the magnitude of an association. Therefore, the data must be interpreted carefully. In addition, this study is that serum samples were not collected from each patient during hospitalization for consecutive days. Because the concentrations of CRP, A1AT and A2MG levels could be changed with time after inflammatory stimulation and differently altered along with the time period [38]. A final limitation of the present study was that there was no histopathologic confirmation of AP, but histopathology can be invasive and expensive, and need general anesthesia in sick dogs [38].

Taken together, our study showed that dogs with AP had low serum concentrations of A2MG and A1AT and high serum concentrations of CRP. AP survivors showed increases in the serum concentrations of A2MG and A1AT, and decreases in the levels of CRP. Therefore, these findings indicate that serum antiproteases and CRP concentrations might be associated with the mortality rate of AP in dogs. In addition, the magnitude of the decrease or increase in serum concentrations of A2MG, A1AT, and CRP might be useful in monitoring clinical progression and response to therapy in dogs with AP. Further studies to confirm these results are required.

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