博士論文

論文題目

Investigation on the novel marker of the dynamic nutritional status and intestinal mucosa integrity in dogs

（犬の動的栄養状態および消化管粘膜健常度に関する新規評価法の検討）

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General Introduction

Malnutrition has been defined as a pathologic state resulting from a relative or absolute deficiency or excess of one or more essential nutrients. The causes of malnutrition include malabsorption, inability to take in nutrients, and hypermetabolism (Young, 1988). The prevalence of malnutrition among hospitalized patients has been well documented in human medicine, especially for surgical patients and critically ill patients (Bistrian et al., 1975; Mullen et al., 1980). Malnutrition causes loss of lean body mass (LBM), mostly muscle mass, and results in impaired immune response and wound healing response to trauma (Young, 1988), increased infectious morbidity and even death (i.e., nitrogen death) (Steffee, 1980) in human. Appropriate nutritional treatment has long been considered important to preserve or normalize lean body mass, and essential for the recovery of critically ill human patients (Roubenoff and Kehayias, 1991). Furthermore, it has been suggested that early recognition of malnutrition and aggressive treatment could reduce the duration of hospitalization and cost (Robinson et al., 1987).

Malnutrition, negative nitrogen balance or net protein loss during hospitalization has been also reported in critically ill dogs and cats (Chan, 2004; Michel, 1993; Michel et al., 2004), Although there is convincing evidence of the deleterious effects of malnutrition in people, the optimal nutritional strategies for critically ill animals remain controversial and

largely unknown. Therefore, recommendations for nutritional support of critically ill animals are generally based on adequate clinical judgment and the limited information available (Chan, 2004).

Nutritional assessment is the most important first step for planning any nutritional treatment (Oka et al., 2006). Good nutritional assessment consists of screening to identify patients at risk, followed by an overall evaluation of the nutritional condition and nutrition planning if necessary (Armstrong and Lippert, 1988; Chan, 2004). The nutritional status of diseased dogs is currently estimated on the basis of body weight (Armstrong and Lippert, 1988; Michel, 1993; Michel et al., 2004), body condition score (BCS) (Michel, 1993; Michel et al., 2004), and several serum biochemical values (Michel, 1993). However, it is a static factor and does not reflect short-term changes. There is few detailed research on the nutritional assessment, especially for critically ill animal patients. In human patients, rapid turnover proteins (RTPs) have recently been used as serum nutritional indicator to reflect the short-term change of nutritional conditions.

RTPs such as transferrin (Tf) and retinol-binding protein (RBP) are metabolized with short half-lives and have relatively small body pool (Fuhrman et al., 2004). These characteristics of RTPs are advantageous for the estimation of short-term nutritional change

and have been used as dynamic nutritional assessment proteins in human medicine (Ingenbleek et al., 1975; Winkler et al., 1989a; Winkler et al., 1989b).

In chapter 1, I evaluated the serial change of plasma Tf and RBP concentrations under experimentally caloric restriction in dogs. Furthermore, plasma Tf concentrations were measured in dogs diagnosed as chronic gastrointestinal disease with or without anorexia to examine its clinical value as a nutritional marker. In chapter 2, I evaluated the relationships between plasma Tf concentration and the nutritional condition and prognosis in malnourished diseased dogs receiving nutritional treatment, and compared with other conventional parameters.

Nutrition treatment/support in veterinary medicine includes oral-assisted feeding, enteral-assisted (tube) feeding, and parenteral nutrition through a catheter that is inserted directly into the veins. Oral- or enteral-assisted feedings are generally recommended first for its beneficial effect on the integrity of gut mucosa (Mohr et al., 2003), which is critical to digest and absorb nutrients. Absorption ability is reported to become remarkably diminished under various situation as intestinal inflammation caused by Crohn’s disease in human patient (D'Agostino et al., 1991b) and experimentally induced injury by (Hughes and Dowling, 1980) methotrexate in rats (Naruhashi et al., 2000). Furthermore, prolonged

parenteral nutritional is also reported to diminish absorption ability because of atrophy in intestinal mucosa in rats (Hughes and Dowling, 1980; Illig et al., 1992). Atrophy of gut mucosa and appended organs is reported in patients with protein malnutrition as well (Hartman et al., 2009).

For nutritional assessment and for adequate planning of nutritional supports of malnourished patients, intestinal integrity should be evaluated as well as the static and dynamic nutritional condition. At present, the intestinal integrity is estimated based on histopathological evaluation using the endoscopic biopsy sample. However, it is not suitable for repetitive evaluation, especially in critically ill dogs due to the need of anesthesia. Further, it is difficult to estimate the function of the whole intestinal tract from histopathological findings using small numbers of biopsy samples.

Diamine oxidase (DAO) is an enzyme performing oxidative diaminating of several polyamines, which inhibit cell proliferation. The activity of DAO is reported to be related to the intestinal mucosa integrity by controlling cell proliferation of villus in human and rats (Wolvekamp and de Bruin, 1994). There is a linear relationship between DAO activities of intestinal mucosa and plasma in patients with intestinal disease (Rokkas et al., 1990). It has been also reported that plasma DAO activity was greatly influenced by nutritional

management and administration of dietary fiber and/or cyclophosphamide (Tanaka et al., 2003). In chapter 3, I measured serum DAO activity in dogs with chronic enteritis and discuss its possibility as a clinical marker to assess intestinal mucosa integrity.

Chapter 1

Usefulness of plasma transferrin concentration as a dynamic nutritional assessment marker in dogs

Abstract

Rapid turnover proteins (RTPs) such as transferrin (Tf) and retinol-binding protein (RBP) has been used as a dynamic nutritional assessment marker in human medicine. The purpose of the present study is to evaluate the clinical usefulness of plasma Tf and RBP concentration as a nutritional assessment marker in dogs. By restricting the total calorie intake to <50% resting energy requirement (RER) in healthy dogs for 2 weeks, plasma Tf concentrations were significantly decreased, which was gradually recovered after bringing the caloric intake back to adequate level. In contrast, plasma RBP and albumin concentrations showed no significant change throughout the study. Next, plasma Tf concentrations were measured in dogs with chronic gastrointestinal diseases. The dogs were divided to 2 groups; <50% RER group, calorie intake <50% RER for more than 1 week; ≥50% RER group, caloric intake ≥50% RER for more than 1 week. Plasma Tf cocentrations were significantly low in severely anorexic dogs (<50% RER group) compared to non-severely anorexic dogs (≥50% RER group) (P<0.05) while plasma albumin concentrations were not different. Taken together, measurement of plasma Tf concentration would be useful to evaluate short-term nutritional status in dogs.

Introduction

Malnutrition is caused by various factors such as critical illness or insufficient food intake, and is reported to increase the morbidity and the mortality in human patients (Heyland, 2000; Kuvshinoff et al., 1993; Mullen et al., 1980; Smale et al., 1981). Therefore, nutritional support/treatment to prevent malnutrition is now considered essential for the recovery of human patients (Roubenoff and Kehayias, 1991). Adequate nutritional treatments have been reported to improve the postoperative recovery and decrease the mortality in hospitalized patients (Heyland, 2000). Recognition that malnutrition may similarly affect veterinary patients emphasizes the need to properly address the nutritional requirements of hospitalized dogs and cats. However, little information is available about the nutritional requirement of critically ill small animals.

Nutritional assessment is an important first step to determine the patients in need of nutritional treatment and to select the adequate treatment (Oka et al., 2006). At present, nutritional status of diseased dogs are estimated based on body weight change (Armstrong and Lippert, 1988; Michel, 1993; Michel et al., 2004), body condition score (BCS) (Michel, 1993; Michel et al., 2004), and several serum biochemical values such as plasma albumin concentration (Michel, 1993). These parameters are called static nutritional assessment markers that reflect average nutritional status of the past several weeks to months. Body

weight change is the most reliable factor assessing the nutritional condition, however, it does not change in a short duration. Additionally, fluid accumulation in the third space has a direct effect on body weight making the assessment difficult. Although BCS is a convenient and non-invasive method to estimate nutritional status of dogs, it is a subjective marker and also affected by the variability of body size and shape among different breeds. Plasma albumin concentration is commonly used biochemical value to assess nutritional status in dogs (Michel, 1993). Because the half-life of albumin in dog is 8.2 days (Dixon et al., 1953), it is expected to be a short-to medium term nutritional assessment marker. However, plasma albumin concentration is readily influenced by fluid accumulation, hepatic function, and protein loss from small intestine or kidney. In addition, plasma albumin concentration rarely decline unless the malnutrition is in the advanced stage, perhaps in part because of the large capacity of the liver to synthesize albumin (Shetty et al., 1979). As serum nutritional indicator for human patients, rapid turnover proteins (RTPs) such as transferrin (Tf) and retinol-binding protein (RBP) have recently been used as dynamic nutritional markers to reflect the short-term change (Raguso et al., 2003).

Tf and RBP play important roles on iron (Hassanein el et al., 1998; Kalantar-Zadeh et al., 1998; Reddy et al., 1970) or vitamin A transport (Ingenbleek et al., 1975), respectively. Both Tf and RBP are synthesized by hepatocytes, released into the circulation, and metabolized with short half-lives (Tf: 8.8 days; RBP: 12 hours in human) (Winkler et al., 1989a). Because of the relatively small body pool and a rapid turn-over rate (Raguso et al., 2003),

serum RTP such as Tf or RBP concentrations had been expected to decline rapidly along with deterioration of the nutritional condition than albumin. Indeed, serial measurement of RTPs is reported to be a useful marker to monitor nutritional status (Inoue et al., 1995; Winkler et al., 1989b) in human patients. In the field of veterinary medicine, however, the clinical usefulness of plasma RTP concentrations as dynamic nutritional assessment markers have not been studied so far.

In this study, I evaluated the serial change of plasma Tf and RBP concentrations under experimentally caloric restriction in dogs. Furthermore, plasma Tf concentrations were measured in dogs diagnosed as chronic gastrointestinal disease with or without severe anorexia to examine its clinical value as a nutritional marker.

Materials and Methods

Dogs

Thirteen healthy laboratory beagles kept in the animal facility of the Veterinary Medical Center of the University of Tokyo were used as controls to determine the reference range of the plasma Tf and RBP concentrations in dogs.

Plasma samples from 64 dogs referred to the Veterinary Medical Center of the University of Tokyo with chronic gastrointestinal diseases with persisted gastrointestinal symptoms for more than 3 weeks were also used. Dogs were divided into two groups according to the amount of daily food intake estimated by the interview with the owners: (1) ≥50%RER group: caloric intake higher than 50% RER for more than 1 week before the admission date, and (2) <50% RER group: caloric intake lower than 50% RER for more than 1 week. RER was calculated based on body weight as described in below. Dogs with plasma C-reactive protein (CRP) higher than 1 mg/dl, iron deficiency anemia, or liver cirrhosis, were excluded from the study due to the possible influence on the Tf concentration (Fleck, 1989; Kalantar-Zadeh et al., 1998).

Experimental short-term calorie restriction

Short-term calorie restriction was performed on 5 healthy laboratory beagles as follows: (1) calorie increase period, 1.5 × daily energy requirement (DER) for 7 days, (2) calorie reduction period, 0.5 × resting energy requirement (RER) for 14 days, (3) calorie re-increase period, 1.5 × DER for 14 days. Plasma samples were collected on day 0, 3, 7, 10, 14, 17, 21, 24, 28, 31 and 35 at early morning under fasting condition, and used for measurement of plasma Tf, RBP and albumin concentrations. Body weight and body condition score (BCS: 5 point scale) were also monitored during the study. RER was calculated according to the following formula: RER = 30 x weight (kg) + 70, DER = RER × 1.6 (Mark, 2001). In the calorie restriction period, a commercial canine low-energy diet (Hill's prescription Diet r/d Canine, Hill's Pet nutrition, Topeka, KS) was fed to all dogs. In other periods, a maintenance diets (CXD-M, Dechra Veterinary Products, Shropshire, UK) was fed. This study was approved by the Animal Care Committee of the University of Tokyo.

Measurement of plasma Tf, RBP and albumin concentrations

Plasma Tf, RBP and albumin concentrations were measured using plasma collected at fasting condition. Plasma were immediately stored at -20 ℃ after preparation until measurement. Plasma Tf concentrations were determined by using a commercial kit (canine Tf ELISA kit: GenWay biotech, San Diego, CA) according to the manufactures' protocol using 1:50000 diluted plasma samples. The measurement range was 6.25 - 400 ng/mL, the

inter-assay % CVs of within 20 were acceptable. Plasma RBP concentrations were determined by using a commercial kit (human RBP ELISA kit: Immundiagnostik, Bensheim, Germany) according to the manufactures' protocol using 1:400 diluted plasma. The measurement range was 1.1 - 33 μ/L and the inter-assay % CVs of within 10 were acceptable. The incubation time with the chromogen substrate was set as 10 minutes for both plasma Tf and RBP concentration measurements. Cross-reactivity between the rabbit anti-human RBP polyclonal antibody and canine plasma RBP was tested by Western blotting according to the previous report (data not shown) (Raila et al., 2000). Plasma albumin concentrations were measured using biochemical analyzer (Dry-chem 7000V: Fuji film CO. Tokyo, Japan). All samples were measured in duplicate.

Statistical analysis

All statistical analysis was performed using SAS software (SAS Institute, Cary, NC). The normality of the data was assessed using the Shapiro-Wilk test. Dunnett's multiple comparison test and Tukey's post hoc tests were used to compare the change of plasma Tf, RBP, and albumin concentrations. The difference of plasma Tf and albumin concentrations between 2 groups were analyzed by *t* test. A second degree polynomial curve that fits the serial data of Tf, RBP and albumin is determined, and a statistical significance value for the curve fit is determined by regression analysis using SAS GLM procedure. Receiver

operating characteristics curve (ROC curve) was used to find out the cut-off line of plasma Tf and ALB which distinguish between <50% RER groups and ≥50% RER groups. Statistical significance was defined as $P<0.05$.

Results

Determination of the plasma Tf and RBP reference range

Plasma Tf and RBP concentrations of 13 healthy dogs were normally distributed according to the statistical analysis. The mean and SD (standard deviation) values were 240 mg/dl and 29 mg/dl for plasma Tf concentration and 0.62 mg/dL and 0.13 mg/dL for plasma RBP concentration, respectively. From these results, the reference range of plasma Tf concentration was determined to be 180 - 300 mg/dL, and that of plasma RBP concentration was determined to be 0.36 - 0.88 mg/dL.

Plasma Tf and albumin concentration in healthy dogs under short-term calorie restriction tests

In test 1, food intake was first increased for 7 days, and then decreased for 14 days followed by 14 days of re-increase period. Plasma Tf concentrations started to decrease after day 7, the beginning of the calorie reduction period. Mean plasma Tf concentration was significantly decreased on day 14 (280 mg/dL), day 17 (250 mg/dL) and day 21 (260 mg/dL) compared to day 7 (350 mg/dL). Plasma Tf concentration increased after day 21 by re-increasing caloric intake, showing no significant difference compared to day 7 (Fig.1).

Although there was no significant difference in plasma Tf concentration at any time point when compared to day 21, the significant (P<0.0001) fit to a second degree polynomial curve was observed during the calorie re-increase period. Plasma Tf concentration under the lower limit of the reference range (<180mg/dL) was observed at none of the time point throughout the experiment. On the other hand, plasma RBP and albumin concentrations were kept within the normal reference range throughout the experiment (Fig. 1), and they were not significantly different at any time point when compared to day 7 at the beginning of caloric reduction period, or day 21 at the beginning of caloric re-increase period. Furthermore, there were no significant fit to second degree polynomial curves in RBP (P=0.1387) and in albumin (P=0.3868). Mean body weight (range; 11.2 to 12 kg) and mean BCS score (range; 3-4) were not significantly different at any time point when compared to day 7 at the beginning of caloric reduction period, or day 21 at the beginning of caloric re-increase period.

Plasma Tf and albumin concentration in dogs with chronic gastrointestinal disease with or without anorexia

Sixty-four dogs diagnosed as chronic gastrointestinal disease were divided to 2 groups (Table 1) according to the amounts of daily food intake: Food intake ≥50% RER (n=42)

without anorexia, and food intake <50% RER (n=22) with anorexia. Breeds included in the ≥50% RER group were Miniature Dachshund (n=5), Chihuahua and Toy Poodle (n=4 each), Japanese Shiba Inu and Pug (n=3 each), Cairn Terrier, Shetland Sheepdog, Miniature Schnauzer, Papillon, Boston terrier and Mongrel (n=2 each), Pembroke Welsh Corgi, Jack Russell Terrier, Labrador Retriever, Hokkaido dog, Yorkshire Terrier, Maltese, Shih Tzu, Borzoi, Italian Greyhound, Miniature Pinscher, Whippet (n=1 each). In the <50% RER group, Miniature Dachshund (n=4), Yorkshire Terrier (n=3), Labrador Retriever (n=2), Jack Russell Terrier, Japanese Shiba Inu, Golden Retriever, Toy Manchester Terrier, Basenji, Beagle, Shetland Sheepdog, Shih Tzu, Chihuahua, Collie, Pug, West Highland White Terrier and Mongrel (n=1 each) were included. Diagnoses of the dogs in the ≥50% RER group include protein losing enteropathy (PLE) in 28 dogs, enteritis without PLE in 10 dogs and other enteropathy in 4 dogs. In <50% RER group, diagnoses include PLE in 15 dogs, enteritis without PLE in 4 dogs and other enteropathy in 3 dogs. Age and sex were not significantly different between the two groups: 21 females (12 intact) and 21 males (11 intact) with median age of 7.08 years (range; 1.16-14.1) in the ≥50% RER group; 9 females (6 intact) and 13 males (9 intact) with median age of 8.70 years (range; 2.00-15.2) in the <50% RER group. The mean body weight was 5.65 kg (range; 1.5-24.8) and 5.45 kg (range; 2-34), and the median BCS score was 3/5 (range; 1-4) and 2/5 (range; 1-3) in the ≥50% RER group and the <50% RER group, respectively.

The mean plasma Tf concentrations in the ≥50% RER group were 200 mg/dl (range; 130-270 mg/dl), and 130 mg/dl (range; 60-200 mg/dl) in the <50% RER group. Plasma Tf concentrations in the <50% RER group were significantly lower compared to that in the ≥50% RER group (P=0.01). Furthermore, 18 dogs (82%) in the <50% RER group and 21 dogs in the ≥50% RER group (50%) showed plasma Tf concentrations lower than the normal limit (180 mg/dl) (Fig. 2A). When the cut-off line of Tf was set as the lower limit of the reference range (180 mg/dl), sensitivity and specificity to predict <50% RER dogs were about 57% and 82%, respectively.

On the other hand, there was no significant difference in mean plasma albumin concentration between ≥50% RER group (mean; 2.6 g/dL, range; 1.8-3.4 g/dL) and < 50%RER group (mean; 2.6 g/dL, range; 1.6-3.6 g/dL) (Fig. 2B).

Discussion

In the present study, plasma Tf and RBP concentrations were measured in dogs and evaluated their clinical usefulness as nutritional markers. First, the reference range of the plasma Tf and RBP concentrations in dogs were determined as 180-300 mg/dL and 0.36-0.88 mg/dL, respectively. The reference ranges of plasma Tf and RBP concentration in human were reported to be 160-360 mg/dL and 3-6 mg/dL, respectively (Winkler et al., 1989b). The reference range of plasma Tf concentrations in dogs was similar to that in human, while the reference range of plasma RBP concentrations was lower than that in humans. Although the reason for the discrepancy is unknown, one possible explanation is that the sensitivity of the ELISA (e.g., lower affinity of monoclonal antibody) used in this study might affect the results of RBP concentration. Another possibility is the difference in vitamin A metabolism between dog and human described below.

In experimental calorie restriction using clinically healthy dogs, significant reduction in plasma Tf concentration was observed during caloric reduction period. On the other hand, plasma RBP and albumin concentration was not significantly different at any time point of caloric reduction period. These results are consistent with the previous report in which plasma Tf but not albumin was decreased in human patients group with low-protein diet (Kopple et al., 1997). In the study of canine anorexic patients, plasma Tf concentration in

the <50% RER group was also significantly decreased compared to the ≥50% RER group while plasma albumin concentration was not different between both groups. From these results, plasma Tf concentration was suggested to be a promising nutritional assessment marker in dogs, reflecting the preceding 1-2 weeks calorie intake, which is more dynamic compared to plasma albumin concentration.

RBP is reported to be a nutritional marker with shortest half-life (0.5 days) among RTPs in humans (Winkler et al., 1989a). Contrary to our expectations, plasma RBP concentration showed no significant change during caloric restriction period in this study. Although RBP is related to the vitamin A transport both in dog and human, the difference in vitamin A metabolism was reported in dogs. Unlike in humans, precursor of vitamin A is transported from liver to the target organs mainly as retinol esters forming complex with lipoproteins (LDL and VLDL) in dogs instead of retinol - RBP complex (Schweigert et al., 1990). The low importance of RBP in canine vitamin A metabolism might be the reason for the negative result in this study.

Dogs with elevated CRP level, iron deficiency anemia, or liver cirrhosis were excluded in this study. Tf is one of the negative acute phase reaction substances, which decreases along with infection or inflammation. Invert correlations between plasma Tf and acute phase proteins such as C-reactive protein (CRP) concentration have been reported in several studies (Fleck, 1989). Liver cirrhosis and iron deficiency anemia are also reported to influence the value of plasma Tf concentrations in human medicine (Kalantar-Zadeh et al.,

1998). Although Tf would be a novel nutritional marker in dogs, these factors should be taken into consideration when assessing the plasma Tf concentration value correctly.

The greatest limitation in this study was the lack of gold standard for the short-term nutritional status in dogs. As body weight and BCS did not change during the restriction period, the relevance of the food restriction protocol is not clear in the calorie restriction study. Furthermore, the amounts of calorie intake in anorexic dogs were estimated according to the interview with the owners. Further study is needed to examine the relation between plasma Tf concentrations and nutritional state by using more accurate data on daily calorie intake. As nutritional status was affected not only by the amount of calorie intake but also intestinal absorption, functional analysis for nutritional absorption should be performed and compared with plasma Tf concentration.

In conclusion, the present study showed that plasma Tf concentration would be useful as a dynamic nutritional assessment marker in dogs. By using plasma Tf concentration in conjunction with the conventional static marker such as albumin, body weight change and BCS, it might be possible to evaluate the objective nutritional state in dogs with malnutriton.

Table 1. Patient population characteristics in the study

		≥50%RER group (*n*=42)	<50%RER group (*n*=22)
Age (years) median		7.08 (1.16-14.1)	8.70 (2.00-15.2)
Sex		21 females (intact 12) 21 males (intact 11)	9 females (intact 6) 13 males (intact 9)
Body weight (kg)		5.65 (1.5-24.8)	5.45 (2-34)
BCS median		3 (1-4)	2 (1-3)
Primary disease in dogs	with PLE	Chronic enteritis (17) Gastrointestinal lymphoma (8) high-grade (5) low-grade (3) Signet ring cell carcinoma (1) Parasitic infection (2)	Chronic enteritis (6) Gastrointestinal lymphoma (8) high-grade (2) low-grade (6) Parasitic infection (1)
	without PLE	Chronic enteritis (7) Gastric adenocarcinoma (1) Antibiotic responsive enteropathy (4) Food responsive enteropathy (2)	Chronic enteritis (5) Gastric adenocarcinoma (1) Carcinoid (1)

PLE: Potein losing enteropathy

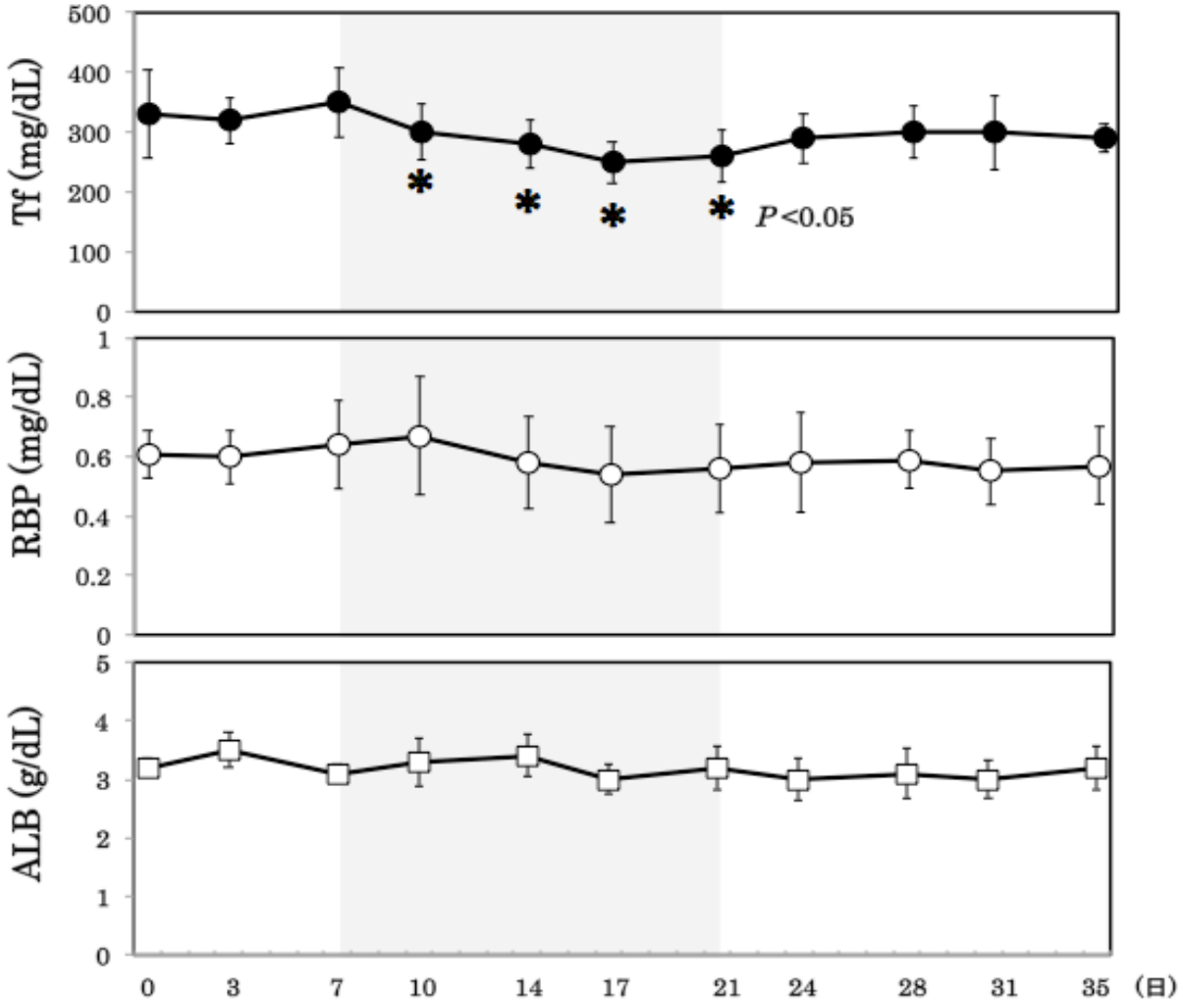

Fig. 1. Time-course change of mean value of plasma Tf concentration (filled circle), plasma RBP concentration (open circle) and albumin concentration (open square) of 5 healthy dogs receiving caloric restriction test according to test 1 protocol described in the Materials and Methods are indicated. An error bar denotes SD value. The amount of food intake was increased for 7 days (day 0 – 7), decreased for 14 days (day 7 – 21, indicated as shadow area) and re-increased for 14 days (day 21 – 35). Asterisks denote the time points with significant difference when compared to day 7 ($P< 0.05$).

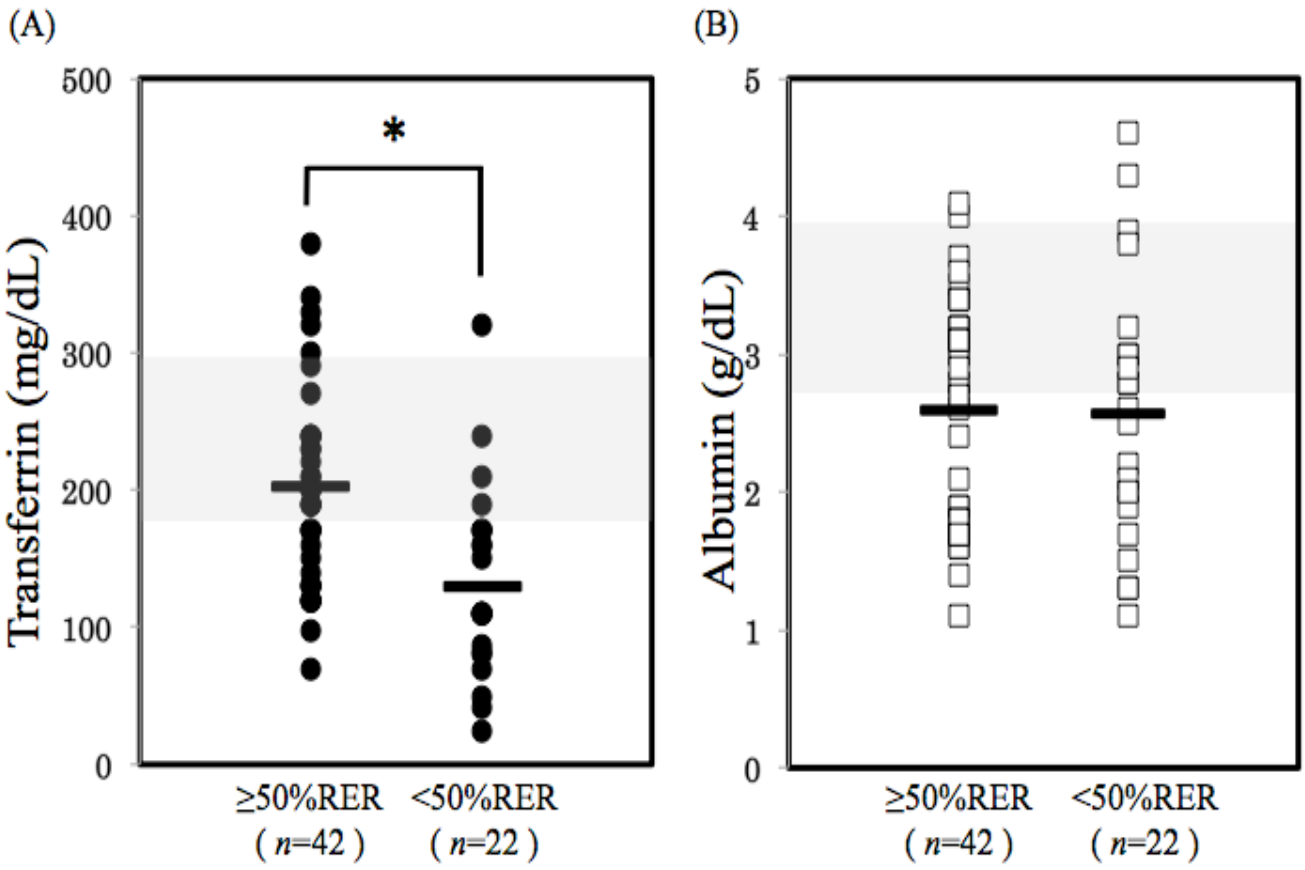

Fig. 2. Plasma Tf concentration (A) and plasma albumin concentration (B) of the dogs with chronic gastrointestinal disease are indicated. Dogs were divided to the ≥50% RER group and the <50% RER group based on the estimated daily calorie intake. A horizontal bar indicates the average value of each group and the shadow area indicates the reference range. Asterisk indicate statistical significance ($P< 0.05$).

Chapter 2

Plasma transferrin concentration as a nutritional marker in malnourished dogs with nutritional treatment

Abstract

Rapid turnover proteins such as transferrin (Tf) are used as dynamic nutritional assessment proteins in human medicine. However, nutritional status in veterinary medicine is mostly assessed on the basis of classical static factors such as body weight, body condition score and plasma albumin level. This study evaluated the clinical usefulness of Tf as nutritional assessment marker by measuring plasma Tf concentrations in malnourished dogs before and after nutritional treatment. Post plasma Tf concentrations were significantly higher than those of pre treatment, although albumin concentration did not change significantly. Numbers of dogs that exhibited increases in plasma Tf concentrations are significantly related to weight gain. Furthermore, the survival rates at day 60 after the treatment initiation were significantly higher in dogs with plasma Tf concentrations above the reference value (180 mg/dL) after the nutritional treatment than those with a plasma Tf concentration <180 mg/dL. In conclusion, the serial measurement of plasma Tf concentration is related to nutritional condition and would be a candidate for novel nutritional assessment marker in dogs.

Introduction

Malnutrition negatively affects the recovery process in various diseases. Compromised immune function and delayed wound healing are reported in humans with malnutrition (Bistrian et al., 1975; Haider and Haider, 1984; Roubenoff and Kehayias, 1991; Seltzer et al., 1979; Steffee, 1980; Thibault and Pichard, 2010; Young, 1988). Effective nutritional therapy improves mortality in malnourished surgical patients or seriously ill patients (Heyland, 2000; Kuvshinoff et al., 1993; Mullen et al., 1980; Smale et al., 1981). Nutritional assessment should be the first step to provide appropriate nutritional treatment (Oka et al., 2006).

In dogs, malnutrition during hospitalization is reported to impair clinical signs and prognosis (Chan, 2004; Remillard et al., 2001). The nutritional status of diseased dogs is currently estimated on the basis of body weight change (Armstrong and Lippert, 1988; Michel, 1993; Michel et al., 2004), body condition score (BCS) (Michel, 1993; Michel et al., 2004), and several serum biochemical values (Michel, 1993). Body weight change is the most reliable factor for assessing nutritional status. However, it is a static factor and does not reflect short-term changes. In addition, fluid accumulation in the third space directly

affects body weight, making assessment difficult. Although BCS is a convenient and non-invasive method for estimating the nutritional status of dogs, it is a subjective marker and is influenced by body size and shape among different breeds. Plasma albumin concentration is a biochemical parameter commonly used to assess nutritional status in dogs (Michel, 1993). As the half-life of albumin is 20 days in humans (Winkler et al., 1989a) and 8.2 days in dogs (Dixon et al., 1953), it is expected to be a short- to medium-term nutritional assessment marker. However, plasma albumin concentration is readily influenced by fluid accumulation, hepatic function, and protein loss from the gut and kidneys. In addition, plasma albumin concentration rarely decreases unless malnutrition is in the advanced stage, possibly in part because of the large capacity of the liver to synthesize albumin (Shetty et al., 1979).

Tf is mainly synthesized in the liver and plays an important role in iron transport (Kalantar-Zadeh et al., 1998). Decreased intestinal protein uptake promptly reduces Tf production, which directly depletes plasma Tf concentration (de Jong et al., 1988; Hassanein el et al., 1998). I previously reported that plasma Tf concentrations are decreased in experimentally induced undernourished dogs, suggesting that Tf may be a dynamic nutritional marker in dogs. Therefore, this study evaluated the relationships between plasma

Tf concentration and the nutritional condition and prognosis in diseased dogs receiving nutritional treatment.

Materials and methods

Dogs and nutritional therapies

Thirty-three dogs referred to the Veterinary Medical Center of the University of Tokyo with clinical signs of anorexia (i.e., taking <50% resting energy requirement [RER] per day over 3 days) were included in this study. Caloric intake was estimated on the basis of interviews with the dogs' owners before nutritional treatment. RER was calculated by the following formula: $RER = 70 \times (\text{body weight in kg})^{0.75}$ (Chan, 2004). All dogs received nutritional treatments which meet the RER of each patient through oral-assisted feeding (OF), enteral-assisted feeding (EF) including esophagostomy, gastrostomy or jejunostomy tube feeding, or parenteral-assisted feeding (PF). The OF and EF dogs except jejunostomy tube feeding were fed mainly semi-digestion liquid diet (CliniCare, Abbott Laboratories) or high density diet (a/d, Hill's Pet Nutrition). For jejunostomy tube feeding, highly digested nutrition agent (Convalescence Support, Royal Canin) was used. The PF dogs received continuous intravenous fluids prepared by mixing dextrose, lipid, and amino acid products to meet RER of each patient. Body weight, BCS, and plasma Tf and albumin concentrations were monitored in all dogs at before and after the nutritional treatment. Dogs with

incomplete medical records or liver failure were excluded from this study.

Subject characteristics

For the purpose of evaluating the relationships between plasma Tf concentration and the effect of nutritional treatment, dogs with pleural effusion and ascites were excluded in order to accurately measure body weight changes. Twenty-one dogs of 33 anorexic dogs were included for this purpose; 9 female (3 intact), and 12 male (6 intact). The median age was 113 months (range: 7–173 months), and median BCS was 2/5 (range: 1–3). The primary disease causing malnutrition was chronic inflammatory gastrointestinal disease in 6 dogs; pancreatitis in 3 dogs; pyloric stenosis in 2 dogs and lymphangiosarcoma, gastric adenocarcinoma, gastric delayed emptying, oral melanoma, and holoprosencephaly, gastrointestinal lymphoma, esophageal diverticulum, megaesophagus, myelodysplastic syndrome, and encephalomyelitis in 1 dog each, respectively. Nutritional treatments performed include OF only (n=11), EF (n=5), PF (n=1), and both EF and PF (n=4).

For the purpose of evaluating the association between Tf and prognosis, neoplastic diseases were excluded and the disease was restricted to the chronic inflammatory gastrointestinal diseases. Twenty dogs histopathologically diagnosed as chronic

inflammatory gastrointestinal diseases were recruited. Nine dogs were female (2 intact), and 11 were male (10 intact). The median age was 105.5 months (range: 58–174 months), and the median BCS was 2/5 (range: 1–3). Fifteen dogs were diagnosed as chronic enteritis, 3 as chronic gastritis accompanying pyloric stenosis, and 1 each as chronic colitis and functional ileus. Six of 20 dogs had apparent ascites due to the hypoalbuminemia as a result of protein-losing enteropathy (PLE). Nutritional treatments included OF only (n=9), EF (n=3), PF (n=6) and, both EF and PF (n=2).

For the purpose of evaluating the serial change of plasma Tf concentrations, one dog with gastrointestinal lymphoma receiving continuous nutritional support (both OF and EF) was monitored. Plasma samples for measurement of Tf and albumin concentration were collected on day 0 (pre treatment), 21, 28, 35, 41, 42 at early morning under fasting condition. Body weight and body condition score were also monitored during the study.

Measurement of plasma Tf and albumin concentrations

Fasting plasma was separated from heparinized blood and immediately stored at −20°C. Plasma Tf concentrations was determined using a commercial kit (Canine Tf ELISA kit: GenWay Biotech, San Diego, CA, USA) according to the manufacturer's protocol; all

samples were measured in duplicate. Plasma albumin concentrations were measured using a biochemical analyzer (Dry-chem 7000V: Fujifilm Co., Tokyo, Japan). Plasma Tf and albumin concentrations were measured at the onset of nutritional treatment (pre-treatment) and after treatment (post-treatment). The interval between both time points ranged from 8–30 days. The reference value for plasma Tf concentration was 180 mg/dL according to the lower limit of the reference range (i.e., mean − 2SD) of healthy dogs as shown in chapter 1. The reference value for plasma albumin concentration was 2.6 g/dL according to the lower limit of our reference range. The survival rates on days 60 after the initial treatment were assessed in each group.

Statistical analysis

Statistical analyses were performed using JMP version 9 (SAS Institute, Cary, NC, USA). The Wilcoxon Signed − Ranks Test was used to compare plasma Tf and albumin concentrations between pre- and post treatment. The Fisher's exact test was used to compare the proportion of dogs categorized by plasma Tf and albumin concentrations and body weight change. The differences of 60-day survival rates after the initiation of nutritional treatment were analyzed using the log-rank test. The level of statistical significance was set

at $P < 0.05$.

Results

Changes in plasma Tf and albumin concentrations before and after nutritional treatment

Plasma Tf and albumin concentrations were compared before and after nutritional treatment. The median plasma Tf concentrations before and after treatment were 150 mg/dL (range: 71–320 mg/dL) and 190 mg/dL (range: 39 – 390 mg/dL), respectively. The median plasma albumin concentrations before and after treatment were 2.8 g/dL (range: 2.1–3.9 g/dL) and 3 g/dL (range: 1.6 – 3.9 g/dL). Plasma Tf concentrations were significantly higher at post-treatment than pre-treatment ($P<0.05$) (Fig.1A). In contrast, plasma albumin concentrations did not differ between pre- and post-treatment ($P=0.5766$) (Fig.1B).

The changes in plasma Tf and albumin concentrations and body weight in each case were subsequently analyzed. Among 15 dogs with increased plasma Tf concentration, 10 dogs showed increases in body weight. In contrast, only 1 of 6 dogs with decreased or unchanged plasma Tf concentration showed increases in body weight. The proportion of dogs that exhibited increases in plasma Tf concentrations was significantly related to weight gain ($P<0.05$) (Table.1). Meanwhile, the numbers of dogs that increased in plasma albumin concentrations were not significantly related to weight gain ($P=0.4663$).

Next, the number of dogs with plasma Tf concentrations above the reference range (i.e., ≥180 mg/dL) were examined. Nine of 11 dogs with increased body weight, 5 of 10 dogs without increase of body weight had ≥180mg/dL plasma Tf concentrations after treatment, respectively. The number of dogs with plasma Tf concentrations ≥180 mg/dL was not significantly related to body weight gain (P=0.1223).

Survival rates 60 days after nutritional treatment initiation

The dogs were divided into 2 groups according to Tf and albumin concentrations: plasma Tf concentrations before or after treatment ≥180 and <180 mg/dL, and plasma albumin concentrations before or after treatment ≥2.6 and <2.6 g/dL. The survival rates 60 days after the initiation of treatment in each group are summarized in Table 2. There was a significant difference in the survival rates 60 days post-treatment between dogs with post-treatment plasma Tf concentrations ≥180 and <180 mg/dL (P < 0.05). The survival rates 60 days post-treatment were also significantly different between dogs with pre-treatment plasma albumin concentrations ≥2.6 and <2.6 g/dL (P < 0.05).

Serial measurement of plasma Tf and albumin concentrations in a dog under nutritional

treatment (Fig. 2)

One beagle dog diagnosed as gastrointestinal lymphoma showed anorexia lasting for several days. Body weight was 8.6 kg and BCS was 2 at day 0. Nutritional support (OF) was started from day 0, when plasma Tf concentration was 210 mg/dL. Plasma Tf concentrations increased to 250 mg/dL and 260 mg/dL on day 21 and 28, respectively. Since the OF became difficult, percutaneous endoscopic gastrostomy (PEG) tube was installed on day 33. Endoscope examination and biopsy of the duodenum was performed concurrently. Histopathological evaluation of the endoscopic biopsy specimen revealed villous atrophy, crypt disappearance and inflammatory cell infiltration in intestinal mucosa together with lymphoma. Although caloric intake by EF had met the RER throughout the observation period, plasma Tf concentration decreased gradually to 170 mg/dL on day 42, which was below the reference value of healthy dog. The dog died on day 42. On the other hand, plasma albumin concentration (mean 2.6 g/dL, range; 2.4-2.8 kg) (Fig. 2), body weight (mean 8.8kg, range; 8.6-9.4kg) and BCS (mean 2/5, range; 2-2) were not changed throughout the nutritional treatment.

Discussion

I previously reported plasma Tf concentrations decrease in both anorexic dogs with various diseases and experimentally induced undernourished dogs. The present results show that plasma Tf concentrations changed depending on nutritional state after nutritional treatment. Furthermore, the results suggest that Tf concentrations after nutritional treatment would be indicative of the prognosis of malnourished dogs with gastrointestinal diseases.

Plasma Tf concentrations increased significantly in malnourished dogs when nutritional treatment was successful, approaching weight gain in many cases. Although the rate of change varied among cases, the variation of plasma Tf concentrations during nutritional treatment could be a useful marker in dogs as shown in chapter 1. In contrast, plasma albumin concentrations did not change significantly between pre- and post-treatment. This is considered to be due to the relatively large body pool of albumin in dogs. Therefore, nutritional condition is not reflected by increased plasma albumin concentration.

The survival rate at day 60 after nutritional treatment was significantly higher in dogs with plasma Tf concentration ≥180 mg/dL. Therefore, the plasma Tf concentration level after nutritional treatment is a candidate prognostic factor for malnourished dogs. In human

medicine, patients with higher serum Tf concentrations have longer survival times than those with lower Tf concentrations (Inoue et al., 1995; Reeds and Laditan, 1976). The present results are concordant with these human studies. Another study showed that an increase in plasma Tf concentration within 4–7 days after the initiation of treatment is associated with better prognosis (Reddy et al., 1970). As the interval between the 2 measurement points (i.e., before and after treatment) varied among cases (range: 12–29 days) in this study, it is necessary to evaluate the appropriate monitoring time of plasma Tf concentrations during nutritional treatment. The reference values used in the present study, 180 mg/dL, was defined on the basis of the data of healthy dogs in my previous study. According to the present data, this reference value may be appropriate when using plasma Tf concentration as a clinical marker of nutritional condition.

Lower plasma albumin concentrations before treatment also influenced the survival rates. Considering that 6 dogs with low plasma albumin concentrations at the onset of treatment were diagnosed with PLE and that hypoalbuminemia is reported to be a prognostic factor of canine chronic enteropathy (Allenspach et al., 2007), the significance of the association between survival rate and albumin concentration before treatment is indicative of the severity of PLE itself. As rapid turnover proteins such as Tf are preserved their blood

levels even in human patients with PLE (Takeda et al., 2003), Tf could be especially useful to assess the nutritional conditions in dogs with PLE.

Plasma Tf concentration was serially monitored in a dog with gastrointestinal lymphoma. Although the calorie intake had met the RER by nutritional treatment, plasma Tf concentration continued to decrease during a week before death. The histopathological evaluation indicated the villous atrophy and crypt disappearance of the intestinal mucosa. In this case, the ability of intestinal mucosa to absorb nutrient might be significantly decreased. It would be important to consider intestinal absorption ability of the dog when deciding the protocol of nutritional support (i.e., OF, EF or PF). Therefore, intestinal integrity and function, as well as nutritional marker such as Tf should be assessed before the initiation of enteral-assisted feeding in critically ill dogs.

This study has several limitations that should be addressed. First, various diseases including malignant tumors were included to evaluate the association between plasma Tf concentrations before and after the nutritional treatment. The type of disease and its severity could affect plasma Tf concentrations. Tf is reported to decrease with infection or inflammation; moreover, plasma Tf and C-reactive protein (CRP) concentrations are reported to be negatively correlated (Fleck, 1989; Krzystek-Korpacka et al., 2008). However,

CRP was not measured in many dogs in the present study. Second, several nutritional therapies including force feeding, enteral and parenteral feeding, and surgery were administered in this study. For example, parenteral intravenous feeding may affect Tf concentrations by altering protein synthesis in the liver. Therefore, additional studies using greater numbers of dogs with a single disease and the same nutritional treatment are required.

In conclusion, to the best of our knowledge, this is the first study demonstrating the clinical usefulness of plasma Tf concentration as a nutrition assessment marker in dogs. Monitoring plasma Tf concentration together with the conventional static markers albumin, body weight, and BCS could be useful for evaluating nutritional status and prognosis during nutritional treatment in dogs.

Table 1. Plasma Transferrin and albumin concentration and body weight change in dog with various primary diseases dogs without pleural effusion and ascites

		Body weight		
		Increased [a] (n=11)	Not change or decreased [b] (n=10)	P value
Transferrin	Increased (n=15)	10	5	0.0382
	Not change or decreased (n=6)	1	5	
Albumin	Increased (n=8)	5	3	0.4663
	Not change or decreased (n=13)	6	7	

Statistically significant by Fisher's exact test

a) Number of dogs with increased body weight after the nutritional therapy.

b) Number of dogs with decreased or unchanged body weight after the nutritional therapy.

Table 2. Plasma transferrin and albumin concentrations and survival rate on day 60 after the nutritional treatment in dogs histopathologically diagnosed as chronic inflammatory gastrointestinal diseases

Evaluation criteria			Survival rate (%)	*P* value
Pre-treatment	Transferrin (mg/dL)	≥ 180 (*n*=5)	60	0.4655
		< 180 (*n*=15)	40	
	Albumin (g/dL)	≥ 2.6 (*n*=9)	78	0.0234
		< 2.6 (*n*=11)	18	
Post-treatment	Transferrin (mg/dL)	≥ 180 (*n*=9)	78	0.0147
		< 180 (*n*=11)	18	
	Albumin (g/dL)	≥ 2.6 (*n*=7)	71	0.1176
		< 2.6 (*n*=13)	31	

Statistically significant by Log-rank test

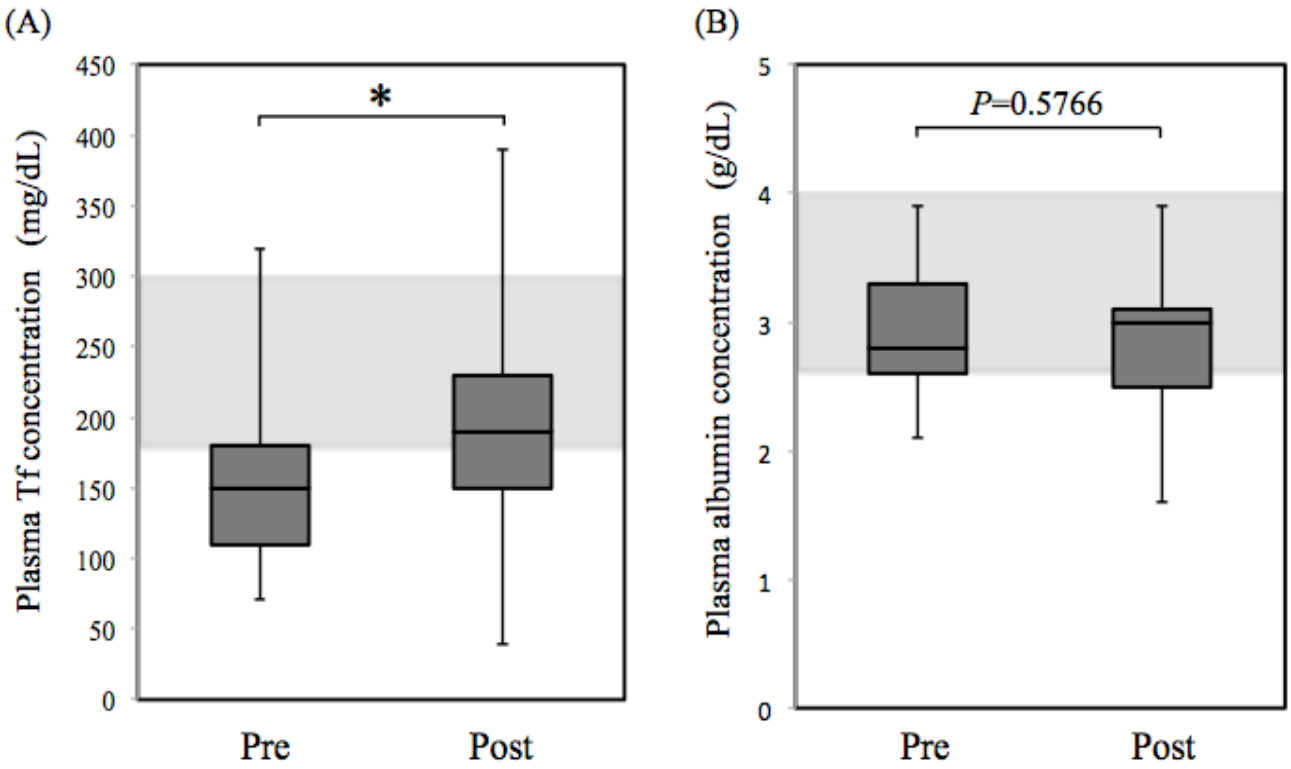

Fig. 1. Plasma transferrin (Tf) (A) and albumin (B) concentrations before and after nutritional treatment. Data are presented as the medican with the 25th-75th percentile range in each box plot. Whiskers indicate the highest and lowest data points. Dotted lines show the lower limit of the reference ranges of plasma (A) Tf (i.e., 180 mg/dL) and (B) albumin (i.e., 2.6 g/dL) concentrations. Pre: pre-treatment, Post: post-treatment, Tf: transferrin. Significant difference was observed between pre- and post-Tf concentration ($P < 0.05$), but was not between pre- and post- albumin concentrations ($P = 0.5766$). The Wilcoxon Signed - Ranks Test was used to compare plasma Tf and albumin concentrations between pre- and post treatment.

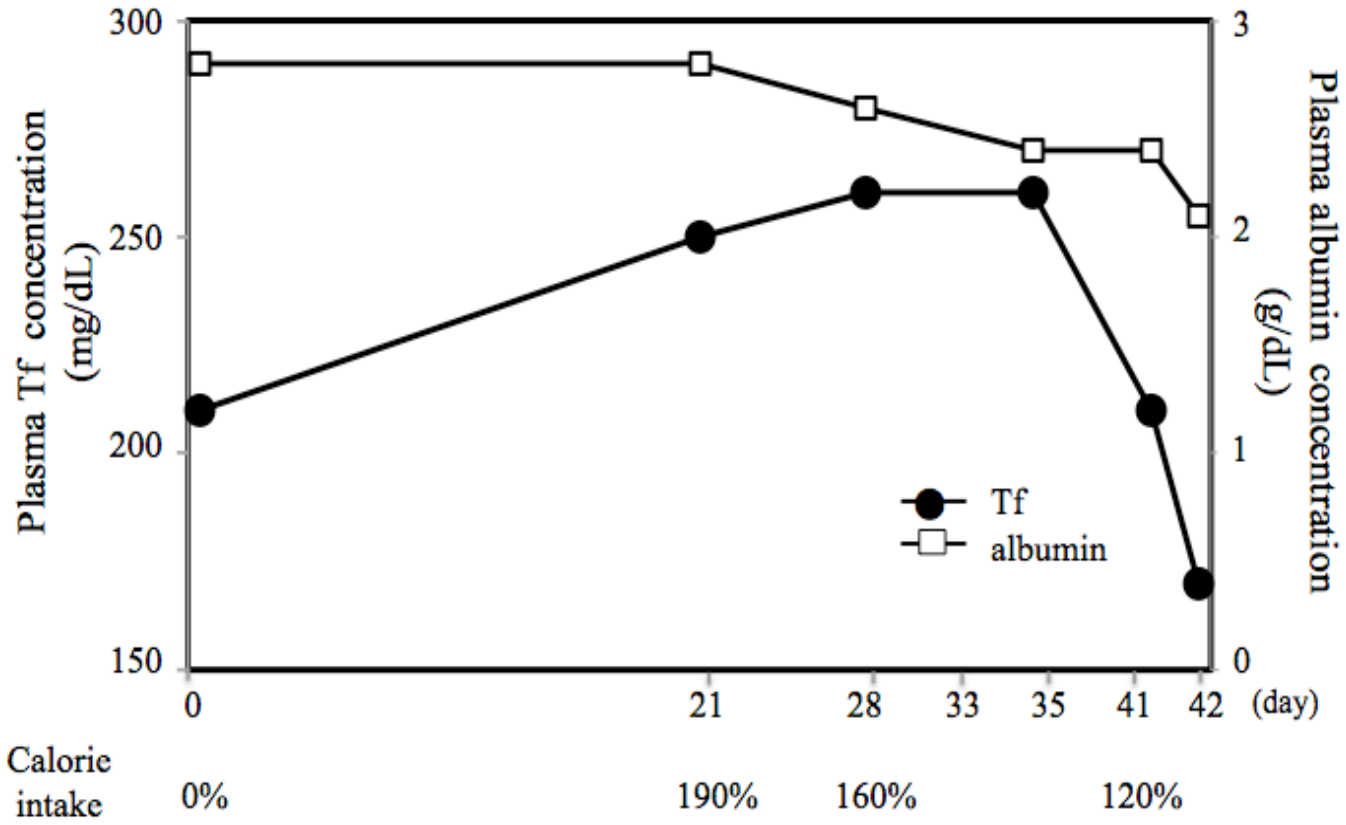

Fig. 2. Serial measurement of plasma transferrin (Tf) (●) and albumin concentration (○) in a dog under nutritional treatment. Caloric intake was estimated on the basis of resting energy requirement (RER).

Chapter 3

Serum diamine oxidase activity in dogs with chronic enteritis

Abstract

Mucosal damage is considered to affect the ability of nutrient absorption causing malnutrition. In order to perform adequate nutritional therapy in dogs with chronic enteritis, assessment of the luminal nutrient absorption ability would be necessary. However, there is no laboratory marker reflecting the mucosal damage or nutrient absorption ability available in dogs at present. The activity of diamine oxidase (DAO) is reported to be closely related to the intestinal mucosal injury and atrophy in human and rats. In this study, I evaluated serum and duodenal DAO activity in dogs with chronic enteritis. Similar to humans and pigs, DAO activity was found to be especially high in the duodenum, colon and kidney in healthy dogs. Significant correlation between serum and duodenal DAO activities was observed in healthy dogs and with chronic enteritis. Further, serum DAO activities in dogs with chronic enteritis were significantly decreased compared to those in healthy dogs, while duodenal DAO activities were not changed between 2 groups. Serum DAO activity was then compared with various clinical parameters including clinical score (CIBDAI), biochemical value and morphological change in duodenal mucosa in dogs with chronic enteritis. Significant correlation was not observed with either of these parameters. However, width of mucosal

villus showed tendency of correlation with serum DAO activity. Serum DAO activity might be related to the status of mucosal villus integrity and further studies to establish clinical significance of DAO would be required in dogs.

Introduction

Malnutrition has been defined as a pathologic state resulting from a relative or absolute deficiency of one or more essential nutrients. The causes of malnutrition include inability to take nutrients, hypermetabolism, and malabsorption (Young, 1988). Intestinal integrity is critical to digest and absorb nutrients. Atrophy of gut mucosa and appended organs is well reported in patients undergoing protein malnutrition. Absorption ability is reported to decline remarkably under various situations. Prolonged parenteral nutritional treatment is also reported to diminish absorption ability of intestinal mucosa in rats because of atrophy (Hughes and Dowling, 1980; Illig et al., 1992). In patients with inflammatory bowel disease (IBD), diminished absorption ability is considered to be a cause of weight loss and specific nutrient deficiency at least in part (Hartman et al., 2009). For the assessment and adequate nutritional planning for malnourished patients and intestinal integrity should be evaluated as well as the nutritional markers.

At present, the intestinal integrity is estimated based on histopathological evaluation using the endoscopic biopsy sample. However, it is not suitable for repetitive evaluation, especially in critically ill dogs due to the need of anesthesia. Further, it is difficult to

estimate the function of the whole intestinal tract from histopathological findings using small biopsy samples. Intestinal permeability and absorption testing using several sugars (i.e., lactulose, rhamnose, xylose, 3-O-methylglucose, sucrose) have been reported as a functional evaluation of mucosal damage in dogs (Allenspach et al., 2006). In the test, urine was collected after intragastric administration of the sugar solution to determine urinary ratio between the absorbed sugars such as lactulose-to-rhamnose (L:R) ratio. The test is, however, time-consuming and also is not suitable for severely ill animals. For the evaluation of intestinal integrities, a novel, non-invasive and convenient methods has been desired in dogs.

Diamine oxidase (DAO) is an enzyme performing oxidative diaminating of several polyamines and is essential for cell proliferation. Diaminating reaction prevents excess absorption of polyamines and histamine originating from ingested food and intestinal bacteria, which inhibit cell proliferation (Wolvekamp and de Bruin, 1994). DAO is abundant in rapidly proliferating tissue and preferentially localized at the tip of villus in small intestine. Intestinal DAO activity is reported to become diminished and to correlate with the severity of histological change in human patients with Crohn's disease (Thompson et al., 1988). It has been also reported that DAO activity of rat intestine decreased along

with villus length in the intestinal mucosa when experimentally injured by methotrexate. Further, absorptive ability evaluated by amino-β-lactam antibiotic cephalexin was shown to be diminished along with the decrease in DAO activity of intestinal mucosa (Naruhashi et al., 2000).

DAO is continuously released from the intestinal epithelia into the lamina propria. There, a portion of them bind to the basolateral membranes of the mucosal epithelial cells, and the remainder is transported into the bloodstream (Wollin et al., 1998). There is a linear relationship between DAO activities of intestinal mucosa and plasma in patients with intestinal disease (Rokkas et al., 1990) as well as in rats with experimentally induced mucosal injury (Luk et al., 1983). Further, the decrease in plasma DAO activity was closely related with the atrophy of injured intestinal mucosa in rats (Luk et al., 1980, 1983). Serum DAO activities have also been shown to decline in patients with malabsorption and villus atrophy due to Crohn's disease (D'Agostino et al., 1987; D'Agostino et al., 1988; Honzawa et al., 2011). Therefore, in human medicine, measurement of serum DAO activity is considered to be a promising candidate for a monitoring marker of mucosal damage in small intestine (D'Agostino et al., 1991a; Wolvekamp and de Bruin, 1994).

In dogs, there is no report about the relationship between DAO measuring and the

intestinal integrity. The purpose of this study is to evaluate the usefulness of serum DAO activity as a clinical marker to assess intestinal mucosa atrophy in dogs with chronic enteritis.

Materials and methods

Dogs

Eleven laboratory beagles were used as healthy controls without any clinical signs of gastrointestinal disease. Six females (4 intact) and 5 males (4 intact) with median age of 65.8 months (range; 19.6-105.7 months) were included. The use of dogs in this study was approved by the Animal Care Committee of the University of Tokyo.

Fifteen dogs diagnosed as chronic enteritis at the Veterinary Medical Center of University of Tokyo were included in this study. Dogs showing either of the chronic gastrointestinal signs (diarrhea, loose stools, vomiting) or hypoalbuminemia were recruited. All dogs were histopathologically diagnosed as chronic enteritis with or without lymphocyte and plasma cell infiltration by using endoscopically biopsied duodenum tissues. Breeds include Miniature Dachshund (n=4), Shetland Sheepdog and Chihuahua (n=2 each), Japanese Shiba Inu, Toy poodle, Miniature Schnauzer, Pekingese, Shih Tzu, Yorkshire Terrier and Pomeranian (n=1 each). Six dogs were female (3 intact) and 9 were male (5 intact). The median age was 68 months (range; 12-158 months), the median body weight was 4.7 kg (range; 1.5-11.95 kg) and median body condition score (BCS) was 3 (range; 2-3).

Body weight, BCS (Michel et al., 2004) and hematological and biochemical values including albumin and C-reactive protein (CRP), known as risk factors for negative outcome in chronic enteropathies in dogs (Allenspach et al., 2007), were recorded in all dogs. Clinical severity of each case was categorized according to the canine IBD activity index (CIBDAI) score (Jergens et al., 2003) at the time of admission. CIBDAI score was determined according to the following clinical status attitude/activity, appetite, vomiting, stool consistency, stool frequency, and weight loss. Scores were summed to yield a total CIBDAI score judged as follows; clinically insignificant (0-3 points), mild (4-5 points), moderate (6-8 points), or severe (>9 points).

Sample preparations

Tissue samples from various organs were obtained from 3 healthy female beagles (median age; 62.8 months, range; 46.4-64 months). All 3 dogs were euthanized for other purpose. Serum DAO concentrations were measured in serum collected at fasting condition. Three endoscopic biopsy specimens were obtained using FB-54Q-1 biopsy forceps (Olympus Medical Systems) from each duodenum for the measurement of duodenum DAO activity and for histological evaluation. For measuring DAO activity, frozen duodenum

tissue samples were homogenized on ice in ice-cold 25 mmol/L phosphate buffer (pH 7.4) using a homogenizer, and then centrifuged at 6000 g for 30 min. The supernatant fraction was stored at -80 ℃ until assay (Naruhashi et al., 2000). For histological study, tissue samples were embedded in paraffin and slides were prepared and stained with H&E.

Measurement of serum and duodenal DAO activity

Measurement of DAO activity in serum and duodenal mucosa were performed based on the methods previously described (Takagi et al., 1994). Briefly, 1.5 ml of substrate cadaverine solution was pre-warmed to 37 ℃ for 5 min, and mixed with 0.1ml of serum or mucosal specimen for exactly 30 min at 37 ℃. The resulting oxidized cataverine was detected by adding 1.5 ml of the coloring reagent (mixed solution of DA-67:Wako, POD; Sigma, ASOD; Wako) and incubating at 37 ℃. The reaction was stopped by incubation for 60 min with 0.05 ml of the stop solution (sodium diethyl dithiocarbamate solution). The calorimetric absorption was measured at 668 nm and 750 nm (Microplate manager ver.5.2.1, BIO-RAD), the latter of which was used as the reference. Blank value was measured concurrently by using water in place of the specimen. DAO activity was quantified using a standard curve prepared from porcine kidney DAO (Sigma Chemical). All samples were run

in duplicate. Results were expressed as enzyme activity /L for serum samples, and enzyme activity /mg protein for duodenum tissue specimen corrected by the protein content determined by the ordinary Lowry method (Lowry et al., 1951).

Measurement of villous length and width in duodenal histopathological specimens

Villi were selected from the histopathological section according to the following criteria for the measurement of length and width. The entire length of the villus should be included in the section, preferably with the lamina propria being visible. The villus tip should be round and contained only a single layer of epithelial cells (Day et al., 2008; Goutal-Landry et al., 2013; Paulsen et al., 2003). Measurement was performed by using calibrated intraocular micrometer. Five to thirty villi per sample were measured, and calculated the average value. The width of the villus was measured in the central part of the villus that measured length.

Statistics

Statistical analyses were performed using a software package (JMP version 9; SAS Institute, Cary, NC). Spearman's rank correlation coefficient was used to examine the relationship between serum DAO activity and duodenal DAO activity, clinical parameters and histopathological parameters. The Mann-Whitney test was used to compare serum and duodenal mucosa DAO activity with healthy dogs and dogs with chronic enteritis.

Results

DAO activities in various canine organs

Tissue homogenate were obtained from various tissue samples of 3 female beagles for measurement of tissue DAO activities. Mean value of DAO activities of each organs were shown in Fig.1. The highest enzymatic activities of each organ were shown in duodenum (0.21 U/mg protein), kidney (0.17 U/mg protein) and colon tissue (0.16 U/mg protein) in dogs, while DAO activities were low in other organs.

Comparison between serum and duodenal mucosa DAO activity in healthy dogs and dogs with chronic enteritis

The relationship between serum and duodenal mucosa DAO activities were evaluated in healthy dogs (n=11) and dogs with chronic enteritis (n=15). Serum DAO activities ranged 0.71-22 U/L, and duodenal mucosa DAO activity ranged 0.02-0.16 U/mg protein. A

significant but weak correlation was found between serum and duodenal mucosa DAO activities (rs=0.3954, $P < 0.05$) (Fig.2).

Comparison of serum and duodenal mucosa DAO activities between healthy dogs and dogs with chronic enteritis

The median serum DAO activity was 12 U/L (range; 6.8-20 U/L) in healthy dogs and 6.6 U/L (range; 0.71-22 U/L) in dogs with chronic enteritis. Serum DAO activities were significantly decreased in dogs with chronic enteritis compared to that of healthy dogs (P=0.0377) (Fig.3A). On the other hand, the median duodenal mucosa DAO activity was 0.087 U/mg protein (range; 0.048-0.14 U/mg protein) in healthy dogs and 0.088 U/mg protein (range; 0.02-0.16 U/mg protein) in dogs with chronic enteritis. Duodenal mucosa DAO activities were not significantly different between both groups (P=0.55) (Fig.3B).

Relevance of serum DAO activity and clinical and histopathological findings in dogs with chronic enteritis

Correlation between serum DAO activity and various clinical and histopathological parameters were evaluated in dogs with chronic enteritis using Spearman's rank correlation coefficient. No significant correlation was found between serum DAO activity and either of the clinical parameters (CIBDAI, CRP, albumin, length / width of villus) evaluated in this study (Table.1). However, serum DAO activity tend to have weak correlation with width of mucosal villus (rs=0.3930, P=0.1473) (Fig.4) and serum albumin concentration (rs=0.3339, P=0.2238) (Table.1) although they did not reach statistical significance. Duodenal DAO activity was also compared with these parameters, and no significant correlation was observed with either of them (data not shown).

In addition, serum and duodenal DAO activities also did not correlate significantly with nutritional markers such as body condition score (BCS) (P=0.8529) and transferrin (Tf) (P=0.7125).

Discussion

To my knowledge, this is the first report to measure serum and duodenal DAO activities in dogs. High DAO activities were observed in the duodenum, colon and kidney, while clearly low activities were observed in other organs. Similar results have been reported in pigs; highest DAO activities in the small intestine and kidney (Klocker et al., 2005). Although DAO activities derived from colon and kidney might affect serum levels of DAO, significant correlation was found between serum and duodenal DAO activities in dogs in this study. Similar results have been reported in human (Rokkas et al., 1990) and rat (Luk et al., 1983), indicating that serum DAO activity is mainly influenced by small intestinal (duodenal) DAO activity. In this study, I did not examine the DAO mucosal distribution at duodenal and cellular location has not been defined in dog. Further immunohistochemical investigations of DAO in dog with antibodies should be need. If serum DAO could reflect the small intestinal integrity, it would be advantageous for serial monitoring especially in severely ill dogs with malabsorption. Although there were no obvious concomitant diseases in the kidney and colon in this study, further investigation should be necessary to determine the effect of these organs on serum DAO activities.

In comparison with healthy dogs, serum DAO activity was significantly lower in the dogs with chronic enteritis. This finding is similar with the results in human IBD patients in whom serum DAO activities decreased with malabsorption and villus atrophy (D'Agostino et al., 1987; D'Agostino et al., 1988; Honzawa et al., 2011). Therefore, measurement of serum DAO activity in dogs could be a candidate for a marker of mucosal damage in small intestine as well as in human. The reduction in number of the duodenal mucosa cells containing DAO might be related with the low serum DAO activity. Various mucosal damage including inflammation, nutritional status, age, and edema in lamina propria occurred in enteritis might cause the reduction of DAO containing cells and resulted in decreased serum DAO activities. However, duodenal DAO activity was not different between healthy and enteritis group. One possible explanation for this discrepancy is the difference of the intestinal area reflected by each samples. Although 3 biopsy specimens were obtained from each dog for duodenal DAO activity measurement, it would be insufficient to reflect the status of the whole intestinal tract. It is reported that DAO activities are different among each samples obtained from different duodenal site (D'Agostino et al., 1984). This would be another reason for that the correlation between serum and duodenal DAO activity is significant but weak in this study.

Unexpectedly, no significant correlation was observed between serum DAO activity and any of the clinical parameters including CIBDAI, CRP and serum albumin concetration, which have been reported to associate with the severity in canine IBD (Allenspach et al., 2007). Similar results have been reported in intestinal absorption/permeability test. Intestinal permeability and absorption testing using several sugars does not correlate with clinical parameters such as CIBDAI or histological score although it shows exacerbation in dogs with chronic enteritis (Allenspach et al., 2006). Furthermore, it is reported that histological change correlates neither with clinical symptoms nor CIBDAI in dogs with chronic enteropathy. All these findings would be also attributed to the limitation of the numbers of endoscopic biopsy as described above. As intestinal absorption/permeability test could not be performed in this study, further investigation is needed to clarify the relationship between DAO activity and intestinal function tests.

DAO is preferentially localized at the tip of villi in small intestine and intestinal DAO activity is reported to decrease along with villus length in experimentally drug-induced mucosal injury. Therefore, villus length and width were measured in dogs with chronic enteritis and also compared with serum DAO activity in this study. I assumed that duodenal mucosa atrophy caused by chronic enteritis results in short and narrow villus, which

correlate with the decrease of DAO activity. In this study, however, width, but not length, of villus showed tendency (*rs*=0.393) of correlation with serum DAO activity. If the sample volume was adequate, statistical significance could be reached based on the scatter plot of serum DAO and width of villus (Fig. 4). So far, clinical significance of villus width has not been known in enteritis. The decreased number of DAO containing cells in villi could affect the width of each villus. Breeds, body weights, non-uniformity of the preparation for histological section, which might affect the length / width of villus, were not considered in this study. Further investigation on alternative objective measuring methods such as crypt-to-villus length ratio with larger sample size is needed to clarify the relationship between DAO and villus architecture.

In conclusion, this is the first study to evaluate the clinical usefulness of DAO activity in dogs with chronic enteritis. Although the clinical value of measuring serum DAO activities did not become clear, DAO could be a novel laboratory marker reflecting small intestinal damage in dogs.

Table 1. Relevance of serum DAO activity and clinical and histological findings in dogs with chronic enteritis

	*rs**	*P* value
CIBDAI	– 0.0236	0.9336
Albumin (g/dL)	0.3339	0.2238
C-reactive protein (mg/dL)	–0.2102	0.452
BCS	0.0524	0.8529
Transferrin (mg/dL)	–0.1039	0.7125
Length of villus (μm)	–0.2681	0.3339
Width of villus (μm)	0.393	0.1473

*Spearman' rank correlation coefficient

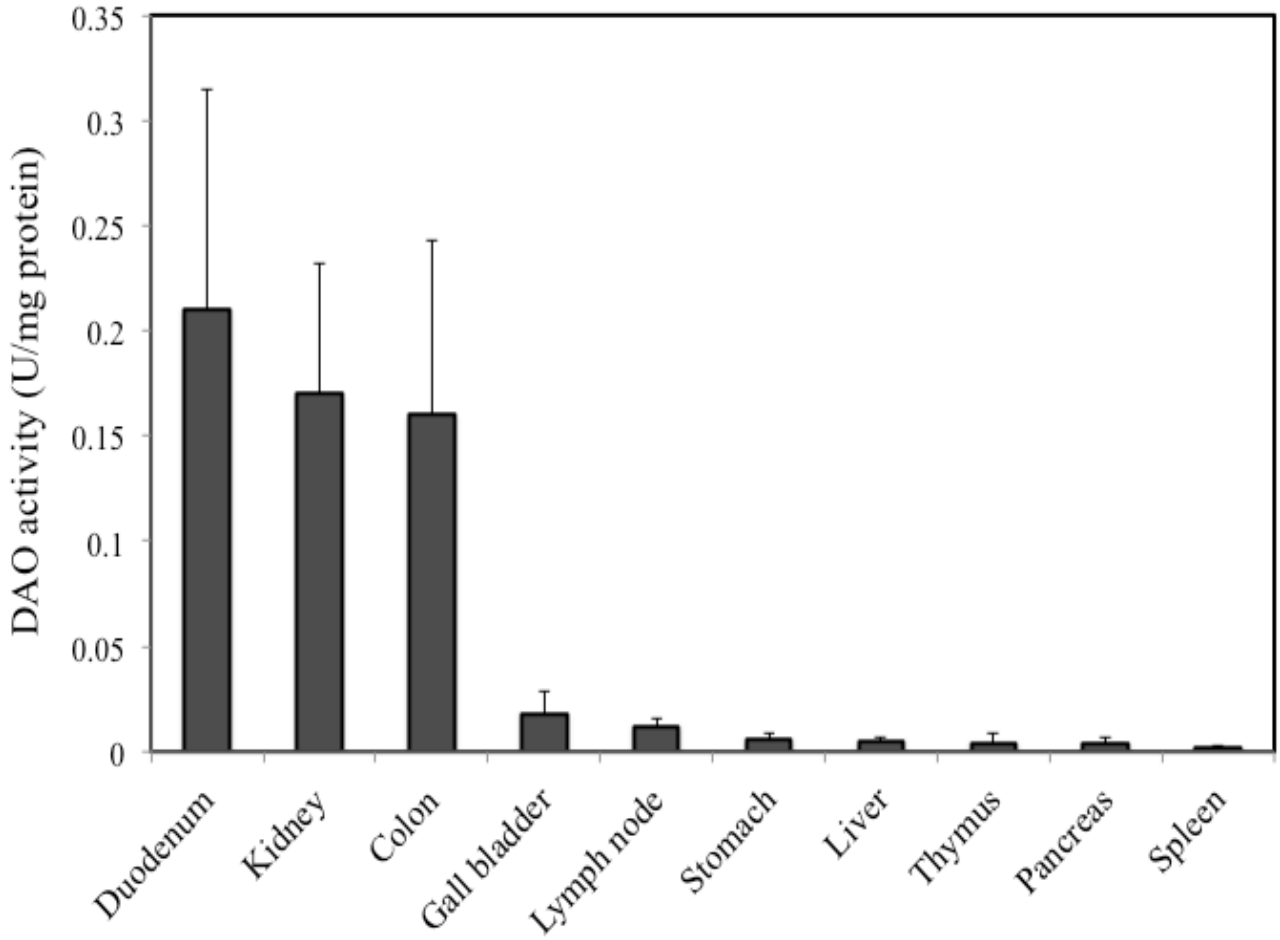

Fig.1. Enzymatic activities of DAO in various organs of healthy dogs. Mean value of 3 dogs was indicated. Error bars indicate standard deviation

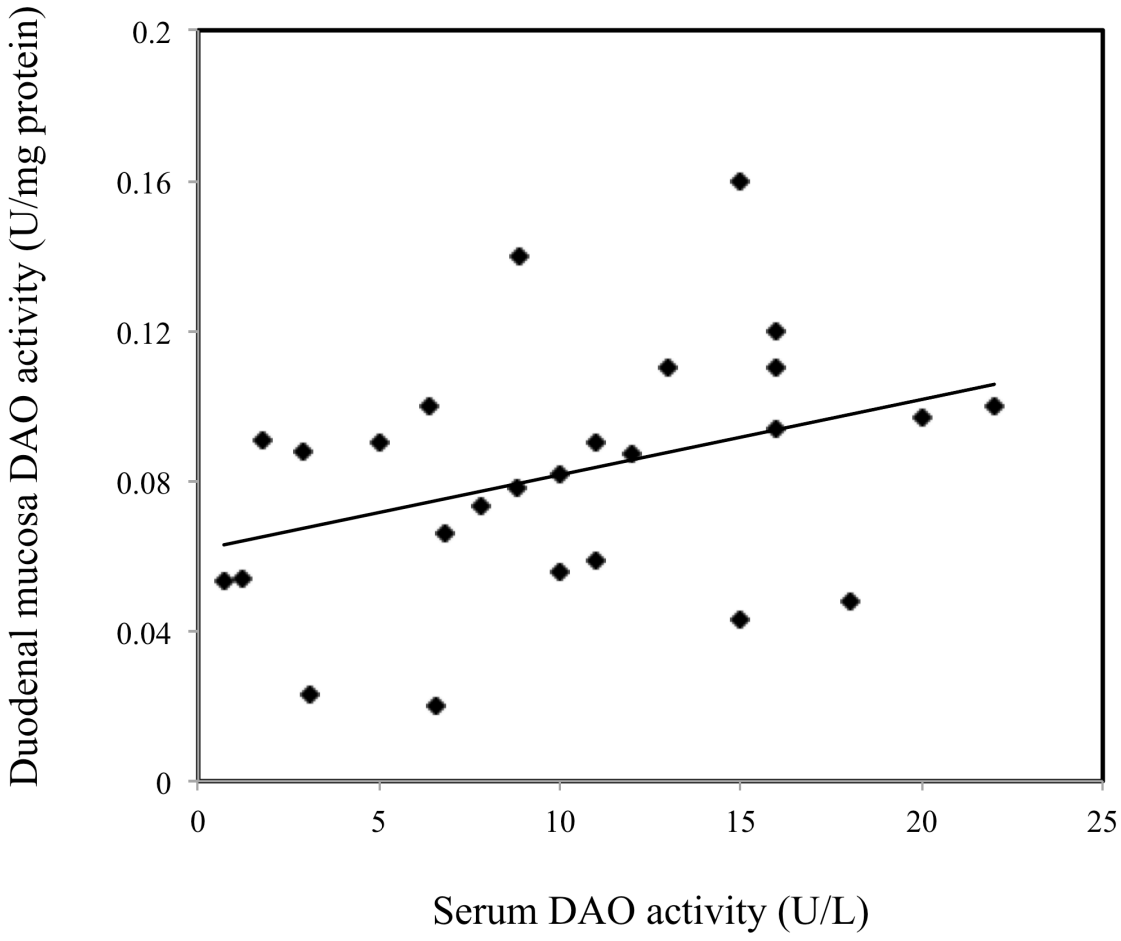

Fig.2. Relation between serum DAO activity (horizontal axis) and duodenal mucosa DAO activity (vertical axis) in dogs (healthy; n=11, chronic enteritis; n=15). Significant correlation was observed when analyzed by using Spearman's rank correlation coefficient (*rs*=0.3954, *P*<0.05).

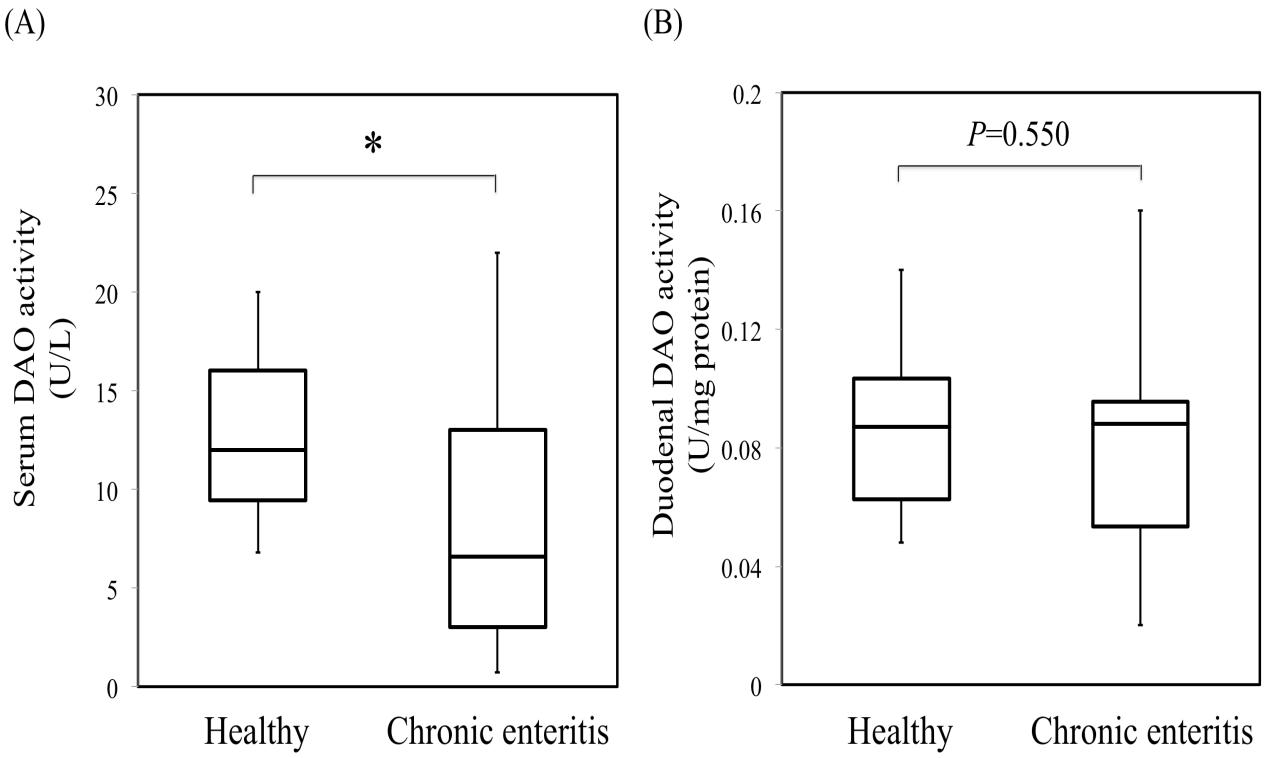

Fig. 3. Comparison of serum (A) and duodenal mucosa (B) DAO activity between healthy dogs (n=11) and dogs with chronic enteritis (n=15). Data are presented as the median with the 25th and 75th quartiles in each box plot. The whiskers indicate the highest and lowest data points. The asterisk indicates statistical significance (P<0.05).

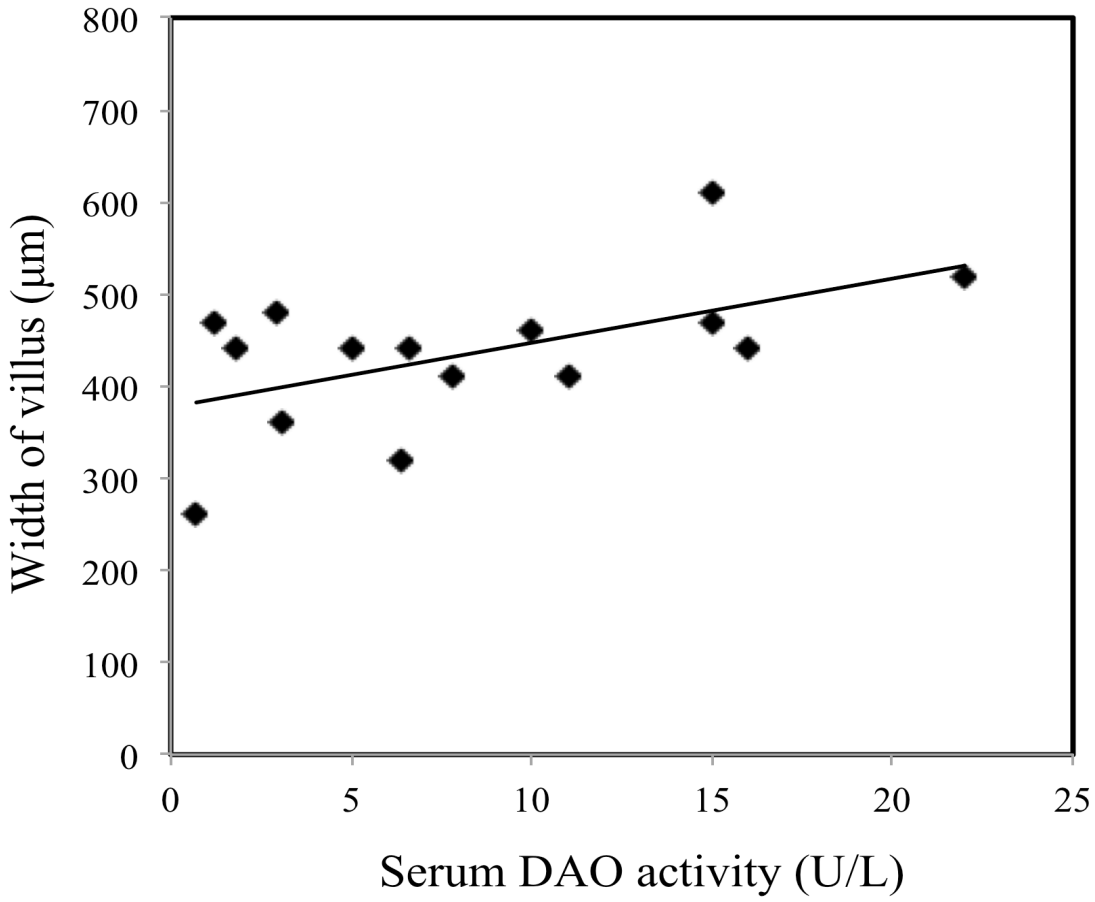

Fig. 4. Relation between width of villus (horizontal axis) and serum DAO activity (vertical axis) in dogs with chronic enteritis (n=15). Although width of villus showed tendency (*rs*=0.393) of correlation with serum DAO activity, statistical significance could not be observed (*P*=0.1473).

Conclusion

Nutritional assessment is the most important first step for planning nutritional treatments. However, nutritional status of diseased dogs is currently estimated on the basis of body weight a body condition score (BCS), and does not reflect short-term nutritional changes. I hypothesized that rapid turnover proteins (RTPs) including tranferrin (Tf) could also be useful in dogs as a dynamic nutritional marker as well as in human.

In Chapter 1, the total calorie intake was restricted to <50% resting energy requirement (RER) in healthy dogs for 2 weeks. Plasma Tf concentrations were significantly decreased, which was gradually recovered after increasing the caloric intake back to adequate level. Furthermore, plasma Tf concentrations were significantly decreased in severely anorexic dogs (<50% RER) compared to non-severely anorexic dogs (≥50% RER group). In Chapter 2, plasma Tf concentrations were measured in malnourished dogs before and after the nutritional treatment. Plasma Tf concentrations were significantly higher after the treatment compared to those before treatment. Numbers of dogs that exhibited increases in plasma Tf concentrations are significantly related to weight gain. The survival rates at day 60 after the treatment initiation were also significantly higher in dogs with plasma Tf concentrations above the reference value (180 mg/dL) after the nutritional treatment than those with a plasma Tf concentration <180 mg/dL. Taken together with the results in Chapters 1 and 2,

plasma Tf concentration would be a useful marker to assess short-term nutritional change in malnourished dogs, especially with nutritional treatment.

Muscle loss, also expressed as decline in lean body mass (LBM), adversely affects immune function and wound healing and is independently associated with mortality in humans (Anker et al., 1997; Freeman and Roubenoff, 1994). Decrease in LBM is usually followed by the decreased rate of protein synthesize in liver, which is possibly reflected as decrease of plasma Tf concentration. To assess the degree of LBM decline or decrease in protein synthesis, measurement of plasma Tf concentration may be useful as an objective marker in dogs. Additional research is needed to investigate the relation between plasma Tf concentration and LBM evaluated by using other methods. In the field of veterinary medicine, muscle condition score (MCS) was developed and recommended for assessing muscle mass (Michel et al., 2011). Scoring MCS is dependent on visual examination and palpation over the temporal bones, scapulae, lumbar vertebrae and pelvic bones. Therefore, MCS is quite subjective and not to be widely used in clinical practice. In human medicine, computer tomography, dual-energy x-ray absorptiometry or deuterium oxide (D2O) dilution method (Son et al., 1998) have been used to estimate LBM. As a clinical marker, measurement of plasma Tf concentration would be more convenient compared with those

parameters which need specialized equipment or anesthesia. In addition, serial and repetitive assessment would become possible by measurement of plasma Tf concentration, which would be helpful especially for planning of appropriate nutritional treatment in hospitalized patients.

Tf is considered to be the RTP with third shortest half-life after Transthyretin (TTR) and RBP in human. Although RBP was not suitable as a nutritional marker in dogs in this study, other RTPs such as human growth hormone (hGH), insulin-like growth factor 1 (IGF-1) or IGFBP-3 would be alternative candidates metabolizing more rapidly than Tf (Baxter et al., 1998; Gianotti et al., 2002). Recently in human medicine, nutritional assessment markers such as TTR and albumin are also used with inflammatory markers including CRP, serum amyloid A (SAA) and α-1 acid glycoprotein to evaluate patient's overall status. Several indices such as Prognostic Inflammatory and Nutritional Index (PINI), Glasgow Prognostic Score (GPS) have been developed and reported to be useful to know the prognosis of each patient (da Silva et al., 2013; Ingenbleek and Carpentier, 1985; Vehe et al., 1991). Although these approaches have not been applied in dogs yet, simultaneous evaluation of both plasma Tf concentrations and CRP would be also useful to monitor the overall status in malnourished dogs.

Serial measurement of plasma Tf concentration in a dog with gastrointestinal lymphoma in Chapter 2 revealed that plasma Tf continued to decrease during a week before death even though the calorie intake had met the RER. Therefore, intestinal integrity and function, as well as nutritional marker should be assessed before the initiation of enteral-assisted feeding in critically ill dogs. At present, the intestinal integrity is estimated based on histopathological evaluation using the endoscopic biopsy sample. However, it is not suitable for repetitive evaluation, especially in critically ill dogs due to the need of anesthesia. In Chapter 3, I evaluated serum and duodenal DAO activity in dogs with chronic enteritis. DAO activity was found to be especially high in duodenum and there was significant correlation between serum and duodenal DAO activities. Serum DAO activities in dogs with chronic enteritis were significantly decreased compared to those in healthy dogs and the width of mucosal villus showed tendency of correlation with serum DAO activity.

In order to assess the usefulness of serum DAO activity as a clinical marker of mucosal integrity, it is necessary to investigate its relevance with intestinal permeability and absorption ability by using sugar absorption test (Allenspach et al., 2006) and measurement of plasma citrulline concentration (Windmueller and Spaeth, 1981). It might be also informative to examine serum DAO activity after vincristine administration, which is known

to have gastrointestinal side effects to dogs (Withrow and David, 2007).

Enteral nutrition (EN) has advantage to maintain intestinal integrity (Mohr et al., 2003) and immunological integrity (Alverdy et al., 1985; Alverdy et al., 1992; Tanaka et al., 1991) and cause bacterial translocation (BT) at lower rate compared to parenteral nutrition (PN) by preventing mucosal atrophy (Gianotti et al., 1994). In contrast, it has been reported that patients of Crohn's disease and patients receiving total PN showed lower plasma DAO activity compared to healthy humans and patients receiving EN. The report also showed that low serum DAO activity seems to reflect atrophy of intestinal mucosa. These previous findings indicate that measuring serum DAO activity is important to estimate mucosal integrity and to determine a adequate nutritional treatment. Although further study is needed, serum DAO activity might be related to and could be a marker for the status of mucosal villus integrity in dog as well as in human.

Even in a recent study, the proportion of hospitalized canine patients in negative-energy balance is reported to exceed 70%. One of the reasons for the insufficient nutritional support is the lack of convenient assessment marker for malnourished dogs. In the series of my thesis studies, I evaluated the usefulness of two laboratory markers; Tf as a nutritional marker and DAO as an intestinal integrity marker. Although these parameters cannot be

applied to veterinary medicine instantly, I hope the results obtained in this study will contribute to the adequate nutritional support for critically ill dogs in the future.

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