Comparison of the expression, activity, and fecal concentration of intestinal alkaline phosphatase between healthy dogs and dogs with chronic enteropathy

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OBJECTIVE
To compare expression, activity, and fecal concentration of intestinal alkaline phosphatase (IAP) between healthy dogs and dogs with chronic enteropathy (CE).

ANIMALS
9 healthy university-owned Beagles and 109 healthy client-owned dogs (controls) and 28 dogs with CE (cases).

PROCEDURES
Cases were defined as dogs with persistent (> 3 weeks) gastrointestinal signs that failed to respond to antimicrobials and anti-inflammatory doses of prednisolone or dietary trials, did not have mechanical gastrointestinal abnormalities as determined by abdominal radiography and ultrasonography, and had a diagnosis of lymphoplasmacytic enteritis or eosinophilic gastroenteritis on histologic examination of biopsy specimens. Duodenal and colonic mucosa biopsy specimens were obtained from the 9 university-owned Beagles and all cases for histologic examination and determination of IAP expression (by real-time quantitative PCR assay) and activity (by enzyme histochemical analysis). Fecal samples were obtained from all dogs for determination of fecal IAP concentration by a quantitative enzyme reaction assay.

RESULTS
For dogs evaluated, IAP expression and activity were localized at the luminal side of epithelial cells in the mucosa and intestinal crypts, although both were greater in the duodenum than in the colon. Active IAP was detected in the feces of all dogs. Intestinal alkaline phosphatase expression and activity were lower for cases than for controls, and fecal IAP concentration for dogs with moderate and severe CE was lower than that for dogs with mild CE.

CONCLUSIONS AND CLINICAL RELEVANCE
Results indicated that dogs with CE had impaired IAP expression and activity. Additional research is necessary to elucidate the role of IAP in the pathogenesis of CE. (Am J Vet Res 2016;77:721–729)

Chronic enteropathy is a chronic idiopathic disorder characterized by persistent clinical signs referable to the gastrointestinal tract with or without histologic evidence of intestinal inflammation. Inflammatory bowel disease is a CE. It is one of the most common causes of chronic vomiting and diarrhea in small animals. Diagnosis of IBD requires the exclusion of all other causes of gastrointestinal clinical signs such as food allergy, exocrine pancreatic insufficiency, infection, and hypoadrenocorticism. The most commonly diagnosed CE in dogs is lymphoplasmacytic enteritis, which is characterized by diffuse infiltration of lymphocytes and plasma cells into the mucosal layer of the intestine with or without architectural changes such as villus atrophy, lymphatic dilation, and epithelial changes. Currently, the pathogenesis of lymphoplasmacytic enteritis is not completely understood.

In human patients, the pathological mechanism of IBD involves an exaggerated or inappropriate host response to the normal gastrointestinal flora or alteration or dysbiosis of that flora. Results of multiple studies suggest that the pathogenesis of CEs in dogs is similar to that for IBD in human patients. The intestinal flora of dogs with idiopathic IBD consists of a significantly greater population of gram-negative bacteria, compared with that of healthy dogs. Dogs with CE frequently have polymorphism and upregulation or downregulation of TLR, a ligand of lipopolysaccha-
ride in gram-negative bacteria. Lipopolysaccharide is an endotoxin, which may have a role in the complex pathogenesis of CE in dogs.

Intestinal alkaline phosphatase is an important factor for homeostatic maintenance of the intestinal flora. It is an isozyme of alkaline phosphatase and is primarily expressed in villus-associated enterocytes and secreted into the feces. Although expression of IAP has been described in humans, rats, mice, and dogs, detailed studies of the sites of IAP expression and secretion into feces have not been performed in dogs. Intestinal alkaline phosphatase is responsible for pH modulation, assimilation of organophosphorus acid, and absorption of fat in the intestinal tract; however, its major function is to act as a barrier for the intestinal mucosa by detoxifying lipopolysaccharide produced by gram-negative bacteria and modulating the binding capability of TLR-4 in enteric bacteria. Abnormalities in IAP expression or function may alter the host barrier against lipopolysaccharide and result in endotoxin-induced inflammation or an aberrant response against the intestinal flora.

In human patients with IBD, IAP expression is negatively associated with severity of IBD. Additionally, oral administration of IAP tablets to mice with colitis resulted in a significant attenuation of colonic inflammation. Consequently, we hypothesized that IAP expression and activity may be altered in dogs with CE. The purpose of the present study was to compare the expression, activity, and fecal concentration of IAP between healthy dogs and dogs with CE.

Materials and Methods

Animals

All study procedures were approved by a research committee and were carried out in accordance with the ethical guidelines of the Tokyo University of Agriculture and Technology. The study consisted of 2 groups of dogs, healthy adult dogs (controls) and adult dogs with CE (cases). The control group consisted of 9 university-owned Beagles and 109 client-owned dogs. Biopsy specimens of the duodenal mucosa and fecal samples were obtained from the 9 healthy adult Beagles that were part of a university research colony.

Sample collection, processing, and storage

Duodenal mucosa and colonic biopsy specimens were obtained from the 9 university-owned control Beagles and 28 dogs in the case group for histologic examination and to determine IAP expression and activity. Briefly, each dog was anesthetized and multiple mucosal biopsy specimens were obtained from the duodenum and colon by use of routine endoscopic procedures. Biopsy specimens intended for histologic examination were fixed in neutral-buffered 10% formalin, whereas those intended for enzyme histochemical evaluation were fixed in neutral-buffered formalin and stored at –80°C until analysis, and those intended for mRNA analysis were stored in RNA-stabilizing tissue storage reagent as directed by the reagent manufacturer’s instructions until analysis.

Histologic examination

Biopsy specimens that were fixed in neutral-buffered 10% formalin were processed in a routine manner and stained with H&E stain. All specimens were histologically evaluated by the same veterinary pathologist, who was unaware of (blinded to) the case or control status of each dog. For each specimen, the extent of intestinal inflammation was scored in accordance with guidelines established by WSAVA and the clinical severity of disease was scored in accordance with the CCECAL.

Enzyme histochemical analysis for IAP

An enzyme histochemical analysis was performed to localize the site of IAP activity in duodenal and colonic mucosa biopsy specimens. Briefly, frozen sections of biopsy specimens were stained with alkaline phosphatase substrate in accordance with the substrate manufacturer’s instructions. That substrate will stain any alkaline phosphatase isozyme; therefore, a section specific inhibitor of IAP, to serve as a negative control. The tissues were then stained with Mayer hematoxylin (1.0 g/mL) to identify cell nuclei.
Enzyme histochemical analysis for IAP-induced lipopolysaccharide dephosphorylation

An enzyme histochemical analysis to detect IAP-induced lipopolysaccharide dephosphorylation activity was performed on biopsy specimens as described. Briefly, routinely prepared frozen sections were fixed in neutral-buffered 10% formalin at 4°C for 10 minutes. The sections were washed and mounted with 50 mM Tris maleate buffer (pH, 7.4) and lead nitrate at 37°C for 60 minutes. The specimens were stained with modified Wachstein and Meisel stain to which magnesium sulfate was added as a catalyzer and lipopolysaccharide was added to act as a substrate for IAP. For each specimen, 2 controls (IAP-inactivated control and negative control) were prepared. For the IAP-inactivated control, L-phenylalanine was added to the stain solution. For the negative control, distilled water was substituted for the lipopolysaccharide in the stain solution. The tissue was then washed with distilled water and reacted with sodium sulfide solution (100 mM) for 60 minutes. The specimens were stained with modified Wachstein and Meisel stain to which magnesium sulfate was added as a catalyzer and lipopolysaccharide was added to act as a substrate for IAP. For each specimen, 2 controls (IAP-inactivated control and negative control) were prepared. For the IAP-inactivated control, L-phenylalanine was added to the stain solution. For the negative control, distilled water was substituted for the lipopolysaccharide in the stain solution. The tissue was then washed with distilled water and reacted with sodium sulfide solution (100 mM) for 60 minutes. Finally, the tissues were stained with Mayer hematoxylin (1.0 g/mL) to identify cell nuclei.

Total RNA isolation and real-time quantitative PCR assay

From each biopsy specimen that was stored in RNA-stabilizing tissue storage reagent, total RNA was extracted with an RNA isolation kit and stored at -20°C until analysis. Primers (Appendix) designed for the detection of a specific sequence of the canine IAP gene mRNA and GADPH gene mRNA (internal control) were used for a real-time quantitative PCR assay as described. The PCR assay was performed in a thermic cycler with SYBR green detection in accordance with the following protocol: 20 seconds at 95°C for 1 cycle followed by 15 seconds at 95°C for 40 cycles, then 30 seconds at 60°C. All PCR assays were performed in duplicate, and the data were analyzed by the relative standard curve method. Results were reported as the ratio of canine IAP gene mRNA to GADPH gene mRNA (IAP expression).

Quantitation of fecal IAP concentration

Fecal IAP concentration was measured by a chromogenic enzyme reaction. Each fecal supernatant sample was thawed and serially diluted with distilled water to obtain dilutions of 1:4,000, 1:8,000, and 1:16,000. A reference solution that contained IAP (1 ng/mL) from the intestinal mucosa of a calf was serially diluted to achieve 10 concentrations ranging from 1:0 to 1:512. The 1:0 concentration (negative control) was distilled water. Then, 100 µL of each diluted fecal supernatant sample or reference solution was added to a well on a 96-well plate. Each dilution of the reference solution was analyzed in duplicate, and to each of those wells, 50 µL of a colorimetric substrate solution that contained p-nitrophenyl phosphate (a substrate specific for alkaline phosphatase) was added. Each dilution of the fecal supernatant samples was analyzed in duplicate under each of 4 conditions. For condition 1, 50 µL of a colorimetric substrate solution was added to each well. For condition 2 (positive control), 50 µL of a colorimetric substrate solution and 100 µL of L-phenylalanine were added to each well; the L-phenylalanine was used to discriminate the colorimetric reaction of other alkaline phosphatase isoenzymes from that of IAP. Conditions 3 and 4 (negative controls) were the same as conditions 1 and 2, respectively, except the colorimetric substrate solution was replaced with distilled water; thus, any colorimetric reaction detected in those wells was the result of the yellowish color of the supernatant samples. The plate was incubated for 30 minutes at 37°C. The optical density of each well was measured at a wavelength of 405 nm by a microplate reader. The mean optical density was calculated for each sample and used for analysis. The optical densities for the serial dilutions of the reference solution were plotted to obtain a standard curve for quantitation of fecal IAP concentration.

Statistical analysis

The difference in IAP expression between the duodenum and colon and the difference in IAP expression between control and case dogs and between case dogs with mild CE (CCECAI score < 5) and case dogs with moderate or severe CE (CCECAI ≥ 5) were evaluated by Student t tests. Fecal IAP concentration and IAP expression in the duodenum were compared between control and case dogs by use of Mann-Whitney U tests. For case dogs, the respective correlations between IAP expression and the WSAVA and CCECAI scores were evaluated by use of simple linear regression. The respective associations between prednisolone administration prior to referral and IAP expression and fecal IAP concentration were evaluated by use of 1-way ANOVA. All analyses were performed with statistical software, and values of P < 0.05 were considered significant.

Results

Control dogs

The 9 university-owned Beagles consisted of 4 males and 5 females with a mean age of 4 years (range, 2 to 7 years). Descriptive statistics for the 109 healthy client-owned dogs were summarized (Table 1).

Case dogs

The 28 dogs with CE included 7 sexually intact males, 12 castrated males, 4 sexually intact females, and 5 spayed females with a mean age of 8 years (range, 2 to 13 years). Breeds represented in the case group included miniature dachshund (n = 6), French Bulldog (3), mixed (3), chihuahua (2), pembroke Welsh Corgi (2), Shiba Inu (2), Shih Tzu (2), and bichon frise, Cairn terrier, Jack Russell Terrier, Old English Sheepdog, Pomeranian, Pug, Shetland Sheepdog,
and Standard Poodle (1 each). Twenty-two case dogs were treated with an antimicrobial either alone or in combination with another antimicrobial prior to examination at the referral hospital. Those antimicrobials included metronidazole (n = 11 dogs), enrofloxacin (5), ampicillin (4), amoxicillin (2), tylosin (2), cefovecin (2), and cefetamet, cephalaxin, clindamycin, erythromycin, olsalazine, orbitoxacin, and oxytetracycline (1 each). Prior to referral, 11 case dogs received anti-inflammatory doses of prednisolone either temporally or sporadically and 12 case dogs received antiemetics, prokinetics, and histamine (H2) receptor antagonists such as metoclopramide (n = 6 dogs), famotidine (5), mosapride citrate (5), ranitidine (3), ondansetron (1), and maropitant (1) alone or in combination. Mucosal lesions observed during endoscopic examinations included villi with a granulated or thickened appearance (n = 10 dogs), edema (7), villi with lymphatic dilation (4), surface irregularities (4), and erosions (1). No gross mucosal lesions were observed during endoscopic examination for 5 dogs.

**Histologic examination**

The histologic diagnosis was lymphoplasmacytic enteritis for 25 case dogs and eosinophilic gastroenteritis for the remaining 3 case dogs. A WSAVA score was assigned to 25 of 28 case dogs. The median WSAVA score for those dogs was 7 (range, 1.5 to 17). A CCECAI score was assigned to 25 of 28 case dogs (the 3 dogs that were not assigned a CCECAI score were not the same 3 dogs that were not assigned a WSAVA score). The median CCECAI score for those dogs was 7 (range, 1 to 18).

**IAP activity and lipopolysaccharide dephosphorylation in duodenal and colonic mucosa**

Alkaline phosphatase activity was identified by the presence of chromogenic reactions on enzyme histochemically stained sections of duodenal and colonic mucosa. Those reactions were effectively inhibited by the presence of L-phenylalanine, which indicated that the observed reactions were the result of activity of the IAP isozyme. In the control dogs, IAP was present on the luminal side of the epithelial cells of the intestinal crypts and villi in the duodenum and mucosal epithelial cells of the colon (Figure 1). The chromogenic reactions were weaker in the colon, compared with those in the duodenum, which suggested that IAP activity was greater in the duodenum than in the colon.

Enzyme histochemical staining of duodenal mucosa biopsy specimens was performed for only 12 of 28 case dogs. The histologic diagnosis was lymphoplasmacytic enteritis for 10 of those dogs and eosinophilic gastroenteritis for the remaining 2. The location of IAP activity in the duodenal mucosa for those 12 case dogs was the same as that for the control dogs (Figure 2). For 6 of the 10 dogs with lymphoplasmacytic enteritis and both dogs with eosinophilic gastroenteritis, the epithelial layer of the duodenal mucosa was much thinner, compared with that of the control dogs. Results of enzyme histochemical staining indicated that the location of lipopolysaccharide dephosphorylation in the duodenum mirrored that of IAP activity in both control (Figure 3) and case dogs.

**IAP expression**

The ratio of canine IAP gene mRNA to GADPH gene mRNA (IAP expression) was determined for the duodenal and colonic mucosa for 6 of the 9 university-owned control Beagles. For those dogs, the mean ± SE IAP expression for the duodenum (0.025 ± 0.006) was significantly (P = 0.022) greater than that for the colon (0.009 ± 0.002). The IAP expression was determined for the duodenal mucosa for the 9 university-owned control Beagles and 26 of 28 client-owned case dogs. The mean ± SE IAP expression in the duodenal mucosal for the control dogs (0.37 ± 0.12) did not differ significantly from that for the case dogs (0.27 ± 0.03). However, when the case group was further separated into dogs with mild CE (CCECAI score < 5) and those with moderate or severe CE (CCECAI score ≥ 5), the mean ±

<table>
<thead>
<tr>
<th>Breed</th>
<th>No. of males</th>
<th>No. of females</th>
<th>Mean (range) age (y)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Miniature Dachshund</td>
<td>12</td>
<td>6</td>
<td>9 (1–14)</td>
</tr>
<tr>
<td>Mixed</td>
<td>8</td>
<td>7</td>
<td>6 (1–18)</td>
</tr>
<tr>
<td>Toy Poodle</td>
<td>7</td>
<td>10</td>
<td>4 (1–10)</td>
</tr>
<tr>
<td>Shih Tzu</td>
<td>6</td>
<td>4</td>
<td>9 (2–17)</td>
</tr>
<tr>
<td>Shiba Inu</td>
<td>2</td>
<td>5</td>
<td>8 (2–15)</td>
</tr>
<tr>
<td>Chihuahua</td>
<td>2</td>
<td>4</td>
<td>6 (3–10)</td>
</tr>
<tr>
<td>Papillon</td>
<td>1</td>
<td>3</td>
<td>10 (3–12)</td>
</tr>
<tr>
<td>Other*</td>
<td>13</td>
<td>19</td>
<td>9 (1–16)</td>
</tr>
</tbody>
</table>

*Breeds represented in this category included Yorkshire Terrier (n = 4), Pug (3), Labrador Retriever (2), Maltese (2), Miniature Schnauzer (2), Shetland Sheepdog (2), West Highland White Terrier (2), and Akita, American Cocker Spaniel, Bichon Frise, Dalmatian, English Cocker Spaniel, Flat-Coated Retriever, French Bulldog, German Shepherd Dog, Golden Retriever, Jack Russell Terrier, Kai-Ken, Norfolk Terrier, Pembroke Welsh Corgi, and Pomeranian (1 each).
SE IAP expression for dogs with moderate or severe CE (0.20 ± 0.04) was significantly (P = 0.022) lower than that for dogs with mild CE (0.42 ± 0.1). The IAP expression was not significantly (P > 0.05) correlated with the WSAVA score (R² = 0.002) and was not significantly (P = 0.61) associated with prednisolone administration prior to referral.

Fecal IAP expression for dogs with moderate or severe CE (0.20 ± 0.04) was significantly (P = 0.022) lower than that for dogs with mild CE (0.42 ± 0.1). The IAP expression was not significantly (P > 0.05) correlated with the WSAVA score (R² = 0.002) and was not significantly (P = 0.61) associated with prednisolone administration prior to referral.

Fecal IAP concentration

Fecal IAP concentration was measured for all control dogs and 11 of 28 case dogs (3 of 6 case Miniature Dachshunds). The mean ± SE fecal IAP concentration for case dogs (19.25 ± 3.09 µg/g) was significantly (P < 0.01) lower than that for control dogs (82.71 ± 5.72 µg/g). Miniature Dachshunds were

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**Figure 1**—Representative photomicrographs of sections of duodenal (A, B, D, and E) and colonic (C and F) mucosa obtained from a healthy adult Beagle (control) following enzyme histochemical staining for IAP with (D, E, and F; negative controls) and without (A, B, and C) l-phenylalanine, an IAP-specific inhibitor. Notice that chromogenic reactions (red) indicative of IAP activity were observed on the luminal side of the epithelial cells of the intestinal crypts close to the intestinal villi of the duodenum (A and B) and mucosal epithelial cells of the colon (C) but were not observed in the negative control slides (D, E, and F). Bar = 100 µm.
the only breed that was sufficiently represented in both the control and case groups to allow breed-specific comparison of fecal IAP concentration between the 2 groups. Similar to the overall study population, the mean ± SE fecal IAP concentration for Miniature Dachshunds in the case group (16.16 ± 9.25 µg/g) was significantly (P < 0.05) lower than that for Miniature Dachshunds in the control group (123.9 ± 15.6 µg/g). The mean fecal IAP concentration did not differ significantly (P = 0.75) between case dogs that did and did not receive prednisolone prior to referral. For the case dogs, fecal IAP concentration was not significantly correlated with either the WSAVA or CCECAI score.

**Discussion**

Results of the present study indicated that, in dogs, IAP expression and activity (lipopolysaccharide dephosphorylation) were localized in and around the luminal side of the epithelial cells of the mucosa and intestinal crypts of the duodenum and colon. Intestinal alkaline phosphatase expression and activity were greater in the duodenum than in the colon. Although the mean IAP expression in the duodenal mucosa did not differ significantly between healthy dogs (controls) and dogs with CE (cases), IAP expression in the duodenal mucosa for dogs with moderate or severe CE was significantly lower than that for dogs with mild CE. Fecal IAP concentration for case dogs was significantly lower than that for control dogs. Collectively, these findings suggested that IAP plays an important role in the pathogenesis of CE in dogs.

For the dogs of the present study, IAP expression (as determined by real-time quantitative PCR assay) and activity (as determined by enzyme histochemical staining for IAP-induced lipopolysaccharide dephosphorylation with (A; negative control) and without (B) L-phenylalanine. Notice that the location of the chromogenic reactions (brown) indicative of lipopolysaccharide dephosphorylation mirrored that for IAP activity in the duodenal sections of Figure 1. Bar = 100 µm. See Figure 1 for remainder of key.
and in the epithelial cells of the mucosa and crypts of the small intestine than in the large intestine.5

When the mucosal biopsy specimens for the dogs of the present study were examined with high-power microscopy, IAP particles associated with epithelial cells had a drop-like appearance. In mice, IAP is connected to the cellular membrane of epithelial cells by glycosylphosphatidylinositol, which is a drop-like molecule.21 If the same is true for dogs, the linkage with glycosylphosphatidylinositol may have accounted for the drop-like appearance of the IAP particles observed in the present study. However, further research is necessary to determine whether IAP particles are linked to epithelial cells by glycosylphosphatidylinositol in dogs.

In the present study, the IAP concentration was measured in fecal samples by an enzyme substrate chromogenic method. Chromogenic reactions were identified in all fecal samples evaluated. Those reactions were inhibited in the presence of 1-phenylalanine, an IAP-specific inhibitor. This suggested that IAP was present in an active form in the feces of dogs. Intact and active IAP is also present in the feces of other animals such as humans and rodents.9 In rats, lysophosphatidylcholine, which is generated after consumption of a meal by hydrolysis of phosphatidylcholine in bile, specifically enhances the release of IAP from enterocytes into the intestinal lumen.22 It is unknown whether IAP is secreted solely or is released in conjunction with the shedding of mucosal epithelial layers, and this warrants further investigation.

Results of the present study indicated that, in dogs, IAP was capable of the dephosphorylation of lipopolysaccharide by reacting with the phosphate residue of lipid A in a manner similar to that observed in rats of other studies.9,22 Lipid A comprises a specific region within the lipopolysaccharide molecule and is responsible for that molecule's endotoxic properties. Dephosphorylation of lipopolysaccharide neutralizes its endotoxic properties21,23–26 and represents an important mechanism in the protection of the intestinal mucosa from the harmful effects of endotoxin.15,23,26,27

Studies27,28 involving mice indicate that IAP inhibits invasion of the intestinal mucosa by bacteria and binds to the lipopolysaccharide ligand TLR-4. Compared with the intestinal flora of healthy dogs, the intestinal flora of dogs with IBD contains significantly greater populations of gram-negative bacteria such as Enterobacteriaceae,29 Proteobacteria,3 Proteus,3 and Acinetobacter5 and lower populations of gram-positive bacteria such as Clostridia.5 Lipopolysaccharide is a component of the outer cell membrane of gram-negative bacteria; therefore, it is likely that dogs with IBD have a greater lipopolysaccharide load in their intestines than do healthy dogs. Results of the present study indicated that IAP expression and lipopolysaccharide dephosphorylation activity of dogs with CE were less than those of healthy dogs. Collectively, those findings suggested that dogs with CE have a greater intestinal load of lipopolysaccharide and an impaired ability to neutralize it. In rodents, IAP regulates and modulates lipid absorption, pH, and intestinal immune function in addition to neutralization of lipopolysaccharide.12,23 Additional research is necessary to determine whether IAP has similar functions in the intestines of dogs.

The mean fecal IAP concentration for healthy control dogs was significantly greater than that for case dogs for the overall study population as well as for Miniature Dachshunds specifically. The IAP expression in the duodenal mucosa for control dogs was also greater than that for case dogs, although that difference was not significant. However, the IAP expression for dogs with moderate or severe CE was significantly lower than that for dogs with mild CE. Intestinal alkaline phosphatase is not dissolved during digestion6; therefore, fecal IAP concentration should reflect the original amount of IAP expressed or secreted by intestinal epithelial cells. Differences in IAP expression between controls and cases and between cases with varying disease severity could have 2 possible explanations that may or may not be part of the same pathogenic continuum. The first explanation is that CE-induced intestinal inflammation results in a decrease in IAP production. The second explanation is that a decrease in IAP production causes intestinal inflammation by impairing lipopolysaccharide dephosphorylation and neutralization, which increases exposure of the intestinal mucosa to active endotoxin and results in an inflammatory response. The fact that the IAP expression for dogs with moderate or severe CE was significantly lower than that for dogs with mild CE suggested that the decrease in IAP expression was the result of inflammation-induced epithelial cell damage. In an in vitro study,50 exposure of an enterocyte cell line to interleukin-1β and tumor necrosis factor-α inhibited expression of the IAP gene. Multiple studies31–34 have been conducted to investigate the expression of various cytokines in the intestinal tissue of dogs. In one of those studies,31 expression of tumor necrosis factor-α in the duodenal mucosa of German Shepherd Dogs with IBD that had small intestinal bacterial overgrowth was significantly greater than that in healthy German Shepherd Dogs. Additional research is necessary to elucidate the effect of proinflammatory cytokine expression and IAP expression in dogs. Because of the small number of cases, comparison of fecal IAP concentration between cases and controls within a specific breed was limited to Miniature Dachshunds. The results for that breed mirrored those for the overall study population and suggested that secretion of IAP is impaired in dogs with CE.

In another study,3 oral administration of recombinant IAP to rats with colitis resulted in significant attenuation of colonic inflammation. Further research is necessary to determine whether all dogs with CE or certain subgroups of dogs with CE would benefit from oral administration of IAP.
The gene that encodes for the expression of IAP is the same gene that encodes for expression of corticosteroid-induced alkaline phosphatase, and in dogs, oral administration of glucocorticoids results in increased expression of IAP. For the dogs of the present study, IAP expression was not associated with the short-term administration of anti-inflammatory doses of prednisolone prior to referral. It is possible that prednisolone, the glucocorticoid most commonly administered to dogs with CE, may increase or at least maintain IAP production during intensive anti-inflammatory treatment.

In the present study, IAP expression and activity were localized in and around the luminal side of the epithelial cells of the mucosa and intestinal crypts of the duodenum and colon of both healthy control dogs and dogs with CE, although the expression and activity of IAP were greater in the duodenal mucosa than in the colonic mucosa. Intestinal alkaline phosphatase that was both intact and active was excreted in the feces of all dogs. In general, IAP expression and activity were lower for dogs with CE than those for control dogs, and fecal IAP concentration for dogs with moderate and severe CE was significantly lower than that for dogs with mild CE. Those findings suggested that IAP has a role in the pathogenesis of CE that is most likely associated with its capability to neutralize endotoxins produced by gram-negative bacteria within the intestinal tract. Additional research is necessary to elucidate the function of IAP in the pathogenesis of CE and explore alternatives for IAP modulation that may facilitate disease treatment.

Acknowledgments

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Footnotes

a. RNAlater, Life Technologies Japan, Tokyo, Japan.


c. Sigma-Aldrich, St Louis, Mo.

d. Wako Pure Chemical Industries Ltd, Osaka, Japan.
e. Illustra RNA Spin Mini RNA Isolation Kit, GE Healthcare Japan, Tokyo, Japan.
f. PrimeScript RT reagent kit, Takara Bio Inc, Shiga, Japan.
g. Thermal Cycler Dice Real Time System II, Takara Bio Inc, Shiga, Japan.
h. SYBR Premix Ex Taq II, Takara Bio Inc, Shiga, Japan.
j. p-Nitrophenyl phosphate liquid substrate system, Sigma-Aldrich Co, St Louis, Mo.
k. iMark microplate reader, Bio-Rad Laboratories Inc, Hercules, Calif.
l. GraphPad Prism 6, MDF Co, Tokyo, Japan.

References


**Appendix**

Primers designed for real-time quantitative PCR analysis to determine IAP expression.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>GAPDH</td>
<td>TCACCGGATTGCGCGATTTG</td>
<td>TGAAGGGGTCAATTGATGGCG</td>
</tr>
<tr>
<td>IAP</td>
<td>CTGGCCCTTGCCATCATC</td>
<td>GATGCGCTCAGCTCTTCTTAG</td>
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The internal control used in the assay was glyceraldehyde-3-phosphate dehydrogenase (GAPDH).