Expression of apical junction complex proteins in duodenal mucosa of dogs with inflammatory bowel disease

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Objective—To determine the expression of tight junction and adherens junction proteins in duodenal mucosa samples of dogs with inflammatory bowel disease (IBD).

Animals—12 dogs with IBD and 6 healthy control Beagles.

Procedures—Duodenal mucosa biopsy samples were endoscopically obtained from dogs with IBD and healthy control Beagles. The expression of claudin-1, -2, -3, -4, -5, -7, and -8; E-cadherin; and β-catenin in the duodenal mucosa samples was determined by means of immunoblotting. The subcellular localization of E-cadherin in the duodenal mucosa samples was determined with immunofluorescence microscopy.

Results—The expression of each claudin and β-catenin was not significantly different between control dogs and dogs with IBD. However, expression of E-cadherin was significantly lower in duodenal mucosa samples of dogs with IBD than it was in samples obtained from healthy control dogs. Results of immunofluorescence microscopy indicated decreased intensity of E-cadherin labeling in the tips of villi in duodenal mucosa samples obtained from 6 dogs with IBD, compared with staining intensity for other dogs.

Conclusions and Clinical Relevance—Results of this study indicated expression of claudin-1, -2, -3, -4, -5, -7, and -8 and β-catenin was not significantly different between duodenal mucosa samples obtained from control dogs and those obtained from dogs with IBD. However, E-cadherin expression was significantly lower in the villus epithelium in duodenal mucosa samples obtained from dogs with IBD versus samples obtained from control dogs, which suggested that decreased expression of that protein has a role in the pathogenesis of IBD in dogs. (*Am J Vet Res* 2014;75:746–751)

Inflammatory bowel disease in dogs is a group of disorders characterized by persistent or recurrent gastrointestinal tract signs and histologic evidence of intestinal inflammation. The disease is a common cause of chronic vomiting and diarrhea in dogs and must be differentiated from other possible causes such as infection, food allergy, neoplasia, exocrine pancreatic insufficiency, and hypoadrenocorticism.[1–3] The most frequently detected form of IBD in dogs is lymphocytic-plasmacytic enteritis, characterized by the diffuse infiltration of lymphocytes and plasma cells into the lamina propria of the intestines. The cause of IBD in humans and dogs is not definitively known. However, factors that may be important to pathogenesis of IBD include changes in mucosal barrier functions and mucosal immune system dysfunction with loss of tolerance to endogenous microflora or dietary antigens, resulting in chronic inflammation of the gastrointestinal tract.[4,5]

The epithelial lining of the gastrointestinal tract creates a barrier that separates luminal contents from

Abbreviations

AJ	Adherens junction
AJC	Apical junctional complex
ALB	Albumin
CCECAI	Canine Chronic Enteropathy Clinical Activity Index
CD	Crohn's disease
IBD	Inflammatory bowel disease
TJ	Tight junction
WSAVA	World Small Animal Veterinary Association

Received January 30, 2014.
Accepted April 14, 2014.
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Supported by a Grant-in-Aid for Scientific Research (No. 23780315) from the Japan Society for the promotion of Science.
Presented in part as a poster at the 2013 American College of Veterinary Internal Medicine Forum, Seattle, June 2013.
The authors thank Dr. M. Inaba for technical support.
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tissue compartments. This barrier function of the intestinal epithelial cell monolayer is regulated by the AJC, consisting of apical TJs and subjacent AJs.[6] Defects in the AJC may cause the high mucosal permeability detected in patients with IBD.[6] Aberrant epithelial barrier function is important in the pathophysiology of IBD in humans.[7] The altered mucosal barrier function influences physiologic immune responses by increasing exposure of immune cells to bacteria and intestinal luminal antigens, which can contribute to the unsuppressed immune response that initiates IBD and worsens outcomes.

Tight junctions create a primary barrier to the diffusion of solutes and water through intestinal paracellular pathways and maintain cell polarity as a boundary between the apical and basolateral aspects of plasma membranes.[8] A major component of TJ strands are the integral membrane proteins, claudins. Claudins are a family of proteins with over 20 homologous subtypes; the differential expression and properties of these proteins are believed to determine tissue-specific variations in electrical resistance and paracellular ionic selectivity among epithelia.[9,10] Adherens junctions are composed of the transmembrane protein E-cadherin and the associated cytoplasmic proteins, the catenins; these are important in the formation of TJs.[11] Various types of derangements in TJs and AJs have been identified in the intestinal epithelium of humans with IBD. Other investigators[12] determined that colonic mucosa in patients with CD has increased expression of claudin-2 and decreased expression of claudin-5 and -8, compared with colonic mucosa in humans without that disease. In addition, results of another study[13] indicate reduced expression of E-cadherin in the colonic mucosa of patients with IBD.

Recently, we determined the protein expression and distribution of several claudins, E-cadherin, and β-catenin in the duodenal and colonic mucosa of healthy Beagles.[14] However, no information has been determined regarding the expression of these TJ and AJ proteins in the duodenal mucosa of dogs with IBD, to the authors' knowledge. The objective of the study reported here was to determine the expression of various TJ proteins (claudin-1, -2, -3, -4, -5, -7, and -8) and AJ proteins (E-cadherin and β-catenin) in duodenal mucosa samples obtained from dogs with IBD.

Materials and Methods

Animals—Dogs (n = 12) referred to Hokkaido University Veterinary Teaching Hospital for evaluation of chronic gastrointestinal tract signs were recruited for this study. A diagnosis of IBD was made on the basis of clinical signs (vomiting, diarrhea, and weight loss) of at least 3 weeks' duration, exclusion of other causes of chronic gastrointestinal tract signs, and detection of lymphoplasmacytic inflammation during histologic examination of duodenal biopsy samples. Food-responsive diarrhea was ruled out by a lack of complete remission of clinical signs during 2 weeks of feeding an elimination diet. Antibiotic-responsive diarrhea was ruled out by a lack of complete response to 2 weeks of administration of metronidazole (10 to 15 mg/kg, PO, twice daily). All dogs with a diagnosis of IBD were scored with the CCECAI for assessment of the severity of the disease.[15] The total CCECAI score was classified as grade 1 (score, 0 to 3), grade 2 (score, 4 to 5), grade 3 (score, 6 to 8), grade 4 (score, 9 to 11), or grade 5 (score, ≥ 12). Written owner consent was obtained for inclusion of dogs in the study. Additionally, all procedures for these dogs were performed with the approval of the Animal Care and Use Committee, Graduate School of Veterinary Medicine, Hokkaido University.

Control tissue samples were obtained endoscopically from 6 Beagles in a research colony (6 sexually intact females; age, 2 years) with a median body weight of 10.5 kg (range, 9.1 to 12.0 kg). None of the dogs had clinical signs of weight loss for more than 1 year before the endoscopic procedure. Hematologic, serum biochemical, fecal, and abdominal ultrasonographic examinations were performed for all dogs. Use of dogs in this study was approved by the Laboratory Animal Experimental Committee, Graduate School of Veterinary Medicine, Hokkaido University.

Tissue sample collection—Duodenal mucosa biopsy samples were endoscopically obtained from the 12 dogs with IBD and 6 control Beagles. Prior to the endoscopic biopsy procedure, dogs were sedated with midazolam (0.01 mg/kg, IV) and butorphanol tartrate (0.02 mg/kg, IV). Then, anesthesia was induced with propofol (4 to 6 mg/kg, IV) and maintained with inhalation anesthesia (isoflurane in oxygen). Gastroduodenoscopy was performed during anesthesia with a flexible video endoscope,[a] and multiple (8 to 10) mucosal biopsy samples were obtained from each of the descending duodenum and caudal duodenal flexure by use of nonserrated biopsy forceps.[b] During the endoscopic procedures, ECGs, respiratory rates, rectal temperatures, arterial blood pressures, pulse oximetry values, and capnography values were monitored and recorded. All endoscopic procedures were completed within 2 hours. Each of the 6 mucosal biopsy samples obtained from the descending duodenum and caudal duodenal flexure was used for histologic examination. Biopsy samples for histologic examination were fixed in neutral buffered 10% formalin, embedded in paraffin wax, and routinely processed for H&E staining. Biopsy samples were obtained from each dog for immunofluorescence microscopy and immunoblot analysis; these samples were snap frozen in liquid nitrogen with and without OCT compound,[c] respectively. Slides of biopsy samples were histologically evaluated by 1 pathologist (YS) who was unaware of the groups and scored on the basis of histologic standards established by the WSAVA Gastrointestinal Standardization Group.[16] In this standard for the assessment of duodenal mucosal samples, 5 morphological features (villous stunting, epithelial injury, crypt distention, lacteal dilation, and mucosal fibrosis) and 3 types of infiltrated leukocytes (intraepithelial lymphocytes, lamina propria lymphocytes, and lamina propria neutrophils) were selected and scored from 0 to 3 in accordance with the guidelines. The total WSAVA composite score was classified as grade 1 (score, 0 to 4), grade 2 (score, 5 to 9), grade 3 (score, 10 to 14), grade 4 (score, 15 to 19), or grade 5 (score, ≥ 20).

Immunoblot analysis—Immunoblot analysis was performed as described with modifications.[14] One biopsy sample of the duodenal mucosa for each dog was homogenized with a plastic pestle in 800 µL of lysis buffer containing 50mM Tris-HCl (pH, 7.4), 1mM EDTA, 2% SDS, and a protease inhibitor cocktail.[d] Then, lysates were incubated for 30 minutes on ice and passed through a biopolymer-shredding system.[e] Protein concentration was determined by the Bradford method with a protein assay kit[f] that included bovine serum ALB as a standard. Aliquots (approx 10 µg) of proteins were separated with PAGE and transferred to a polyvinylidene difluoride filter,[g] followed by blocking with 2% nonfat milk, incubation with rabbit polyclonal antibodies against claudin-1, -2, -3, -5, -7 and -8 (polyclonal antibody designations: JAY.8, MH44, Z23.JM, Z43.JK, ZMD.241, and ZMD.446, respectively; final concentration, 0.08 µg/mL diluted in 2% nonfat milk),[h] a mouse monoclonal antibody against claudin-4 (clone 3E2C1; 0.17 µg/mL),[h] mouse monoclonal antibody against E-cadherin (clone 36/E-cadherin; 0.04 µg/mL),[i] mouse monoclonal antibody against β-catenin (clone 14/Beta-catenin; 0.06 µg/mL),[i] or mouse monoclonal antibody against β-actin (clone AC-74; 0.02 µg/mL)[j] for 2 hours. Membranes were then incubated with horseradish peroxidase–conjugated swine anti-rabbit IgG polyclonal antibody (0.11 µg/mL)[k] or goat anti-mouse IgG polyclonal antibody (0.5 µg/mL)[l] for 1 hour. Signals were detected with a chemiluminescent detection reagent.[m] Densitometric analyses of immunoblot images were performed with software.[n] The intensity of the signals of each TJ and AJ protein was normalized to the corresponding β-actin signal.

Immunofluorescence microscopy—Frozen sections (thickness, 7 µm) of duodenal mucosa samples were fixed with 95% ethanol at 4°C for 30 minutes, followed by 100% acetone at room temperature (approx 24°C) for 1 minute. Sections were incubated with 1% bovine serum ALB in PBS for 20 minutes. Sections were incubated with a monoclonal antibody against E-cadherin (0.8 µg/mL) for 1 hour at room temperature. As a negative control, sections were incubated with control mouse IgG (0.8 µg/mL). Sections were then washed 3 times with PBS, followed by incubation for 30 minutes with goat anti-mouse IgG antibodies conjugated with fluorescent dyes (5 µg/mL).[o] After washing with PBS, sections were embedded in fluorescent mounting medium[p] and examined by use of a laser scanning microscope.[q]

Statistical analysis—Statistical analyses were performed by use of a computer program.[r] Normality of distribution was assessed with the Shapiro-Wilk *W* test; some data were determined to be nonnormally distributed. Expression levels of each protein were compared between the 2 groups by use of the Mann-Whitney *U* test. The relationship between E-cadherin protein expression (normalized to β-actin expression) and the serum ALB concentration and total CCECAI or WSAVA score for IBD dogs was evaluated by use of the Spearman rank correlation coefficient. Values of $P < 0.05$ were considered significant.

Results

IBD dogs—Twelve dogs with a diagnosis of IBD were included in this study. All of these dogs had inflam-

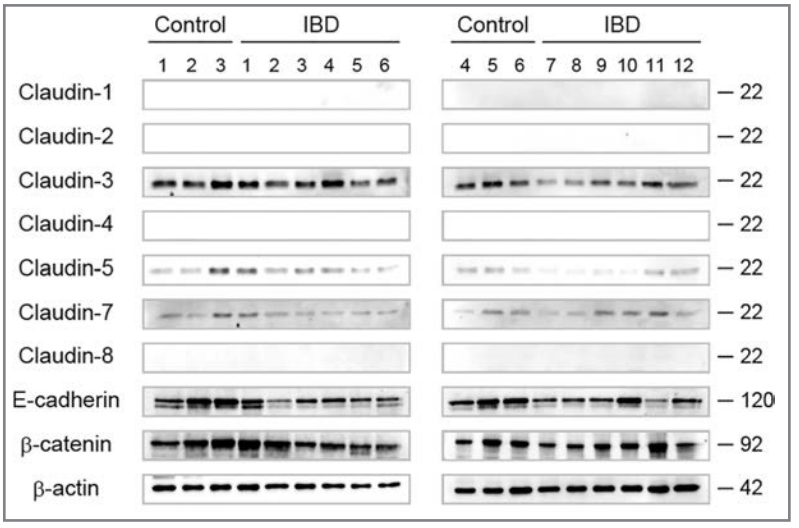

Figure 1—Photographs of immunoblots that indicate expression of various TJ and AJ proteins in duodenal mucosa biopsy samples obtained from 6 control dogs and 12 dogs with IBD; β-actin is included as a loading control. Values on the right side indicate the apparent molecular mass (in kilodaltons) of each protein.

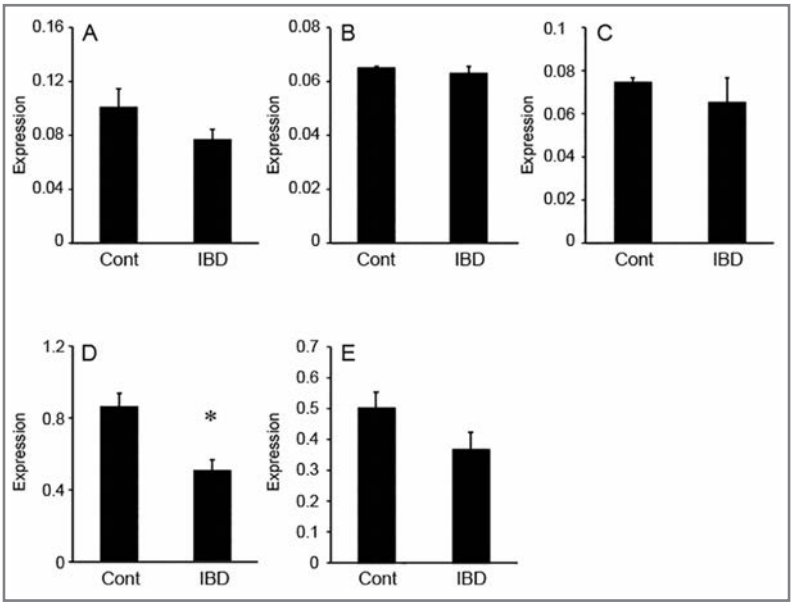

Figure 2—Mean ± SEM expression of claudin-3 (A), -5 (B), and -7 (C) and E-cadherin (D) and β-catenin (E) in duodenal mucosa samples of healthy control dogs (Cont; n = 6) and dogs with IBD (12). Expression units for each protein are relative to β-actin expression. *Value is significantly ($P = 0.019$) different from the value for the control group (assessed with the Mann-Whitney *U* test).

mation in intestinal mucosal samples and a histologic diagnosis of lymphocytic-plasmacytic enteritis. Breeds of dogs included 2 Border Collies, 2 Maltese, and 1 each of Chihuahua, Boston Terrier, Toy Poodle, Pomeranian, Bernese Mountain Dog, Shetland Sheepdog, Beagle, and Jack Russell Terrier. The median age of these dogs was 7 years (range, 2 to 10 years) with 7 females (2 sexually intact and 5 neutered) and 5 sexually intact males. The median body weight of the dogs was 6.15 kg (range, 1.7 to 33.1 kg).The median serum ALB concentration was 1.6 g/dL (range, 1.0 to 2.5 g/dL). The CCECAI scores of the 12 dogs with IBD included 2 dogs with grade 1, 3 dogs with grade 2, 4 dogs with grade 3, 2 dogs with grade 4, and 1 dog with grade 5. The median CCECAI score for dogs with IBD dogs was 6.5 (range, 3 to 17). The total WSAVA scores of dogs with IBD included 7 dogs with grade 1 and 5 dogs with grade 2 (no dogs had scores of grade 3, grade 4, or grade 5). The median total WSAVA score for dogs with IBD was 4 (range, 1 to 8). Prednisolone (2 mg/kg, PO once daily for 2 weeks, followed by a decrease in the dosage every 2 to 4 weeks if a satisfactory response [on the basis of improvement in clinical signs and serum ALB concentration] was detected) was administered to all dogs with IBD after endoscopy; 2 dogs were also treated with cyclosporine (5 mg/kg, PO once daily for > 4 weeks).

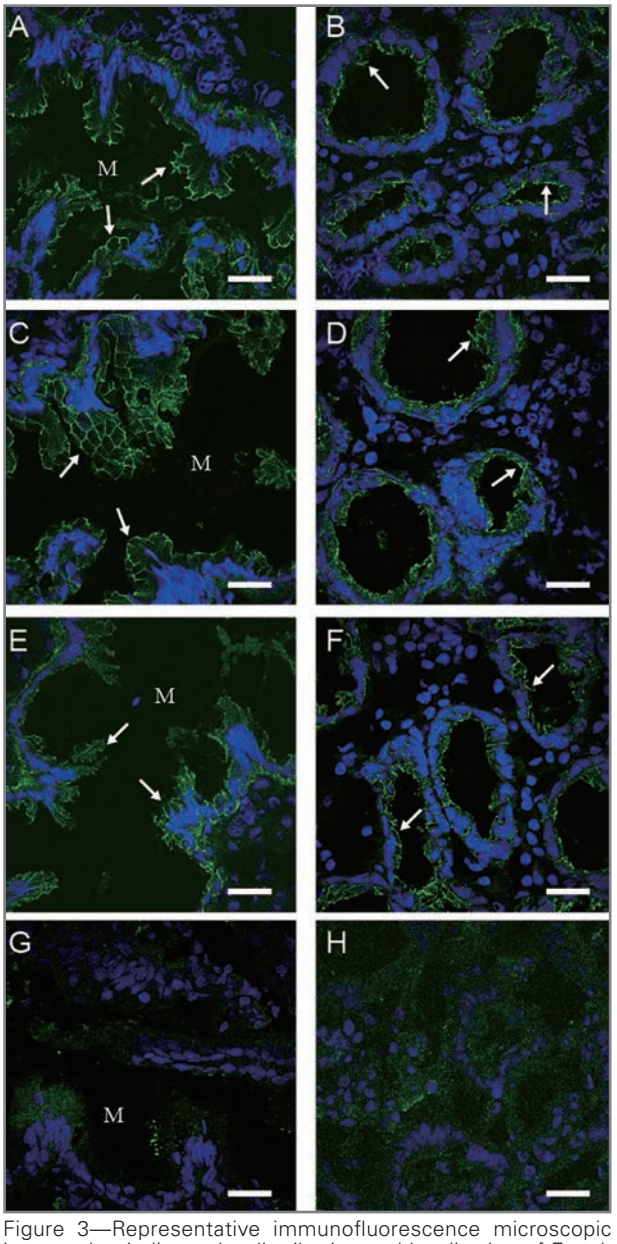

Figure 3—Representative immunofluorescence microscopic images that indicate the distribution and localization of E-cadherin in villi (left column [A, C, E, and G]) and epithelial crypts (right column [B, D, F, and H]) of duodenal mucosa samples obtained from healthy control dogs (A and B), a dog with IBD (C and D), and another dog with IBD (E and F) and in negative control slides (G and H). Arrows indicate AJCs. M = mucosal surface. Bars = 20 µm.

Healthy dogs—Each control dog was determined to be healthy, had no clinical signs of gastrointestinal tract disease, and had no physical examination, laboratory analysis, or abdominal ultrasonographic abnormalities. The median total WSAVA score for duodenal mucosal biopsy samples obtained from the 6 healthy control dogs was 1 (range, 0 to 1; classified as grade 1).

TJ and AJ protein expression in duodenal mucosa samples—Expression of claudin-3, -5, and -7; E-cadherin; and β-catenin proteins were observed in the duodenal mucosa of dogs with IBD and healthy control dogs (**Figure 1**). Expression of claudins -1, -2, -4, and -8 was not detected in duodenal mucosa samples of any dogs. No significant differences in expression of claudin-3, -5, and -7 and β-catenin were detected between duodenal mucosa samples obtained from dogs with IBD and those obtained from control dogs (**Figure 2**). Expression of E-cadherin was significantly lower in duodenal mucosa samples obtained from dogs with IBD (mean ± SD, 0.49 ± 0.31; range, 0.22 to 1.26), compared with expression in samples obtained from healthy control dogs (mean ± SD, 0.85 ± 0.25; range, 0.50 to 1.14). Expression of E-cadherin for dogs with IBD was not significantly correlated with serum ALB concentration (r_s = −0.084; P = 0.78), total CCECAI score (r_s = 0.028; P = 0.92), or total WSAVA score (r_s = −0.029; P = 0.92).

Immunostaining of E-cadherin in duodenal mucosa samples—In duodenal mucosa samples obtained from healthy control dogs, E-cadherin–specific labeling was intense at the epithelial cell AJC (**Figure 3**). Expression of E-cadherin seemed to be uniform along the crypt-to-villus axis in duodenal mucosa samples obtained from healthy control dogs and samples obtained from 6 of the dogs with IBD. In contrast, the fluorescence intensity of E-cadherin staining was weak at the tips of villi in duodenal mucosa samples obtained from 6 of the dogs with IBD.

Discussion

Results of this study indicated alterations in E-cadherin expression in duodenal mucosa samples obtained from dogs with IBD, compared with expression in samples obtained from healthy control dogs. Low E-cadherin immunofluoresence was also observed in villus epithelial cells of duodenum samples obtained from dogs with IBD.

A reduction in E-cadherin expression has been detected in colonic mucosal epithelium of humans with

IBD at the site of ulceration.[17,18] Reduced E-cadherin staining was detected at the mucosal edges around epithelial ulcerations of patients with active ulcerative colitis and CD, although E-cadherin staining was preserved in adjacent nonulcerated colonic epithelial cells.[18] Similar to findings for humans with IBD, dogs with IBD in the present study had reduced E-cadherin expression in the duodenal mucosa. However, ulcerated lesions are rare in dogs with IBD. Also, reduced E-cadherin staining was detected at villus epithelial cells of the duodenum in samples obtained from such dogs in this study. Although it is unknown whether reduced expression of E-cadherin is the cause or result of intestinal inflammation, such reduced E-cadherin expression may have various roles in the induction or persistence of intestinal inflammation in dogs with IBD.

A low immunofluoresence E-cadherin expression signal intensity was observed in duodenal mucosa samples of several dogs with IBD in this study. Low intensity of E-cadherin staining may be caused by villus epithelial morphological changes such as villous shunting and epithelial injury.[16] Low E-cadherin expression might alter the permeability of villous epithelium and lead to malabsorption. However, the strength of E-cadherin staining for some dogs with IBD was not consistent with results of immunoblot analysis. The reason for this discrepancy was not determined; however, immunoblot analysis may yield more accurate quantitative results than immunofluorescence microscopy analysis. In addition, biopsy samples used for immunoblot analysis and those used for immunofluorescence microscopy were not identical; differences in results may have been attributable to the use of different samples for each assay.

For humans with IBD, there is a significant correlation between low expression of E-cadherin and disease activity.[18] In contrast, the amount of E-cadherin expression was not correlated with serum ALB concentration or clinical or histologic severity scores for dogs with IBD in the present study. Humans with ulcerative colitis commonly develop ulcerative lesions in colonic mucosa during an active disease state, and E-cadherin expression may be reduced concurrent with ulcer formation. In contrast, severe ulcerative lesions do not typically develop in the duodenal mucosa of dogs with IBD, regardless of the clinical or histologic severity. In addition, histologic changes in duodenal mucosal biopsy samples are poorly correlated with clinical severity in dogs with IBD.[15,19,20] The poor correlation between E-cadherin expression and disease severity in dogs with IBD may be partly attributable to such histologic features.

The cytoplasmic domain of E-cadherin directly binds to β-catenin, and β-catenin links to the actin microfilament network of the cellular cytoskeleton via α-catenin.[21] This binding is essential for the formation of stable cell-cell adhesion. In contrast to E-cadherin expression, there was no statistically significant difference in β-catenin expression between duodenal mucosa samples obtained from dogs with IBD and those obtained from control dogs in this study. Other authors[18] also reported that β-catenin expression was not altered in colonic mucosal epithelium samples obtained from humans with IBD, although E-cadherin expression was reduced. Thus, expression of E-cadherin might not be associated with expression of the cytoplasmic protein β-catenin.

In another study[14] we conducted, claudin-3, -5, and -7 proteins were detected in duodenal mucosa samples obtained from healthy dogs. In the present study, we also detected claudin-3, -5, and -7 proteins in the duodenal mucosa samples obtained from dogs with IBD. In a study[12] of humans with IBD, investigators identified decreased expression of claudin-3 and -5 in colonic mucosa samples obtained from patients with CD. The expression level of claudin-3 in duodenal mucosa samples obtained from dogs with IBD in the present study seemed to be low but was not significantly different from expression in samples obtained from healthy control dogs. In addition, claudin-5 and -7 expression were not significantly different between dogs with IBD and control dogs. Thus, decreased expression of claudin proteins might be not associated with mucosal inflammation in dogs with IBD.

In another study,[14] we determined that expression of claudin-2, -4, and -8 proteins could not be detected in duodenal mucosa samples obtained from healthy dogs.[14] Similarly, claudin-2, -4, and -8 proteins were not be detected in duodenal mucosa samples obtained from dogs with IBD in the present study. Humans with CD have increased expression of claudin-2 in colonic mucosa.[12] In addition, increased intensity of immunofluorescent staining for claudin-2 is detected in the proximal crypt and luminal surface of colonic mucosa samples obtained from dogs with lymphocytic-plasmacytic colitis, compared with findings for other dogs.[22] In contrast to those findings, claudin protein expression was not higher in duodenal mucosa samples obtained from dogs with IBD in this study, compared with results for control dogs; this result suggested that increased expression of claudin protein in the duodenal mucosa might not be associated with the pathogenesis of IBD in dogs with low WSAVA scores.

Results of a recent study[23] indicate that the expression of several genes associated with maintenance of the intestinal epithelial barrier, including claudin-8, is downregulated in the duodenal mucosa of dogs with chronic enteropathy. Also, humans with CD have lower expression of claudin-8 protein, compared with other humans.[12] However, claudin-8 protein expression was not detected in duodenal mucosa samples obtained from healthy control dogs or dogs with IBD in the present study. The importance of decreased expression of claudin-8 is unknown.

In another study[14] we conducted, claudin-1 expression was faintly detected in duodenal mucosa samples obtained from healthy dogs. In contrast, claudin-1 protein expression was not detected in duodenal mucosa samples obtained from healthy control dogs or dogs with IBD in the present study. This difference in findings regarding claudin-1 expression might have been attributable to differences in antibody dilution, because dilution of the primary antibody was modified in the present study to optimize band density. However, the expression levels of claudin-1 protein are likely low in the duodenal mucosa of healthy dogs and dogs with IBD.

The present study had some limitations. Inflammatory changes in the lamina propria of dogs with IBD leads to dilution of epithelial proteins. In addition, the epithelial surface area could be decreased in dogs with IBD, compared with that in other dogs, because of villus shunting and epithelial injury. These changes may have resulted in underestimation of TJ and AJ protein expression. In addition, the total WSAVA scores for dogs with IBD in this study were low, although dogs with clinically severe disease were included. Additional studies which include dogs with IBD that have high total WSAVA scores would be necessary to confirm results of this study. Also, we performed histologic examination of ileum and colon samples concurrently with examination of duodenum samples for only 5 and 8 of the 12 dogs with IBD, respectively. The discrepancy between CCECAI scores and WSAVA scores for dogs with IBD in this study may have been partly attributable to that study limitation. Furthermore, the healthy control dogs used in this study were not matched for age and sex to dogs with IBD because of the limited availability of healthy control dogs.

Results of the present study indicated E-cadherin protein expression was significantly lower in duodenal mucosa samples obtained from dogs with IBD than in such samples obtained from healthy control dogs. To the authors' knowledge, this is the first study in which TJ and AJ protein expression in duodenal mucosa of dogs with IBD was determined.

a. VQ-8143A, AVS, Tokyo, Japan.
b. FB-53Q-1, AVS, Tokyo, Japan.
c. Sakura Fine Technical Co, Tokyo, Japan.
d. Complete Mini EDTA-free, Roche Diagnostics GmbH, Mannheim, Germany.
e. QIAshredder, QIAGEN, Hilden, Germany.
f. Bio-Rad Laboratories Inc, Hercules, Calif.
g. Immobilon-P, Millipore, Bedford, Mass.
h. Zymed Laboratories Inc, Carlsbad, Calif.
i. BD Biosciences, San Jose, Calif.
j. Sigma-Aldrich Corp, St Louis, Mo.
k. Catalogue No. P 0399, DakoCytomation, Tokyo, Japan.
l. Catalogue No. 610-1302, Rockland Immunochemicals Inc, Gilbertsville, Pa.
m. ECL Prime Western Blotting Detection Reagent, GE Healthcare, Buckinghamshire Little Chalfont, England.
n. LumiVision Analyzer 2.0, Aisin Seiki Co Ltd, Aichi, Japan.
o. Catalogue No. 28176-H48, Hilyte Fluor 488, AnaSpec Inc, San Jose, Calif.
p. DakoCytomation, Tokyo, Japan.
q. LSM Laser Scanning Microscope, Zeiss, Oberkochen, Germany.
r. JMP 8, SAS Institute Inc, Cary, NC.

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