Expression and subcellular localization of apical junction proteins in canine duodenal and colonic mucosa

Hiroshi Ohta, DVM, PhD; Tomoki Yamaguchi, DVM; B. K. Wickramasekara Rajapakshage, BVSc; Masahiro Murakami, DVM; Noboru Sasaki, DVM; Kensuke Nakamura, DVM; Shiang-Jyi Hwang, BVM; Masahiro Yamasaki, DVM, PhD; Mitsuyoshi Takiguchi, DVM, PhD

Objective—To examine the expression and distribution of tight junction (TJ) and adherens junction (AJ) proteins in canine duodenal and colonic mucosa.

Sample—Mucosa obtained from 4 healthy Beagles.

Procedures—Biopsy specimens of the duodenum and colon were obtained via endoscopy from 4 healthy dogs. The expression patterns and subcellular localization of claudin-1, -2, -3, -4, -5, -7, and -8; E-cadherin; and β-catenin in the duodenum and colon were analyzed by use of immunoblotting and immunofluorescence microscopy.

Results—In the duodenum, there was clear expression of claudin-3 and -5, E-cadherin, and β-catenin proteins and weak expression of claudin-7 protein. In contrast, there was clear expression of claudin-2 and -3, E-cadherin, and β-catenin proteins and weak expression of claudin-5 and -7 proteins in the colon, as determined by use of immunoblotting. As determined by the use of immunofluorescence microscopy, the duodenum and colon had staining for claudin-3 and -5, E-cadherin, and β-catenin in the most apical region and staining for claudin-7 in the basolateral region. Staining for claudin-2 was also observed in the colon.

Conclusions and Clinical Relevance—Information was provided about the expression patterns of TJ and AJ proteins in the duodenum and colon of clinically normal dogs. These results may provide valuable information for use in evaluating the importance of these TJ and AJ proteins in the pathogenesis of inflammatory bowel disease in dogs. (Am J Vet Res 2011;72:1046–1051)

In dogs, IBD is a group of disorders characterized by persistent or recurrent gastrointestinal signs and histologic evidence of intestinal inflammation. The disease commonly causes chronic vomiting and diarrhea in dogs and must be differentiated from other possible causes (eg, infection, food allergy, neoplasia, exocrine pancreatic insufficiency, and hypoadrenocorticism) of chronic vomiting and diarrhea. The forms of IBD identified most frequently in dogs are lymphocytic-plasmacytic enteritis and LPC characterized by the diffuse infiltration of lymphocytes and plasma cells into the enteric lamina propria. Lymphocytic-plasmacytic enteritis mainly affects the small intestines, and LPC affects the large intestine. The definitive cause of IBD in humans and dogs is unknown. However, the factors suspected of possibly playing important roles in the pathogenesis of IBD include changes in mucosal barrier functions and dysfunction of the mucosal immune system with loss of tolerance to endogenous microflora or dietary antigens that results in chronic inflammation in the gastrointestinal tract.

The epithelial lining of the gastrointestinal tract creates a barrier that separates luminal contents from the underlying tissue compartments. The barrier function of the intestinal epithelial cell monolayer is regulated by the AJC, which consists of apical TJs and subjacent AJs. Defects in function of the intestinal epithelial barrier have been observed in a number of intestinal disorders, such as IBD. It is becoming evident that aberrant function of the epithelial barrier plays a central role in the pathophysiologic processes of IBD. Alterations in the mucosal barrier influence the physiologic immune response by increasing exposure of immune cells to bacteria and luminal antigens, which in turn can contribute to the unsuppressed immune response that initiates IBD and worsens its outcome.
Tight junctions create the primary barrier to the diffusion of solutes and water through the paracellular pathway and maintain cell polarity as a boundary between the apical and basolateral domains of the plasma membrane. The major components of TJ strands are the integral membrane proteins occludin and the claudins and some cytoplasmic scaffolding proteins (eg ZO-1, which anchors claudins to actin filaments). Claudins are a family of >20 homologous subtypes; the differential expression and properties of these subtypes are believed to determine the tissue-specific variations in electrical resistance and paracellular ionic selectivity among epithelia. Adherens junctions consist of the transmembrane protein E-cadherin and the cytoplasmic protein catenins; AJs play an important role in the formation of TJs. Studies on IBD in humans have identified numerous types of derangement in both TJs and AJs of the intestinal epithelium. Investigators in 1 study detected increased expression of claudin-2 and decreased expression of claudin-5 and -8 in colonic mucosa obtained from patients with Crohn’s disease. In addition, investigators in another study reported reduced expression of E-cadherin in the colonic mucosa from patients with IBD.

Expression of claudin-2, E-cadherin, and β-catenin proteins in colonic mucosa of healthy dogs and dogs with LPC have been evaluated by use of immunofluorescence microscopy. In that study, there was an increase in the expression of claudin-2 in the colonic mucosa from dogs with LPC, which indicated a role for claudin-2 in the pathogenesis of LPC in dogs. However, there is a paucity of information about the expression of TJ and AJ proteins in the duodenum of clinically normal dogs, and qualitative data about expression of the TJ and AJ proteins in the colon of clinically normal dogs are limited. As an initial step in understanding the roles of TJ and AJ proteins in the pathogenesis of IBD in dogs, the study reported here was conducted to examine the expression and distribution of the TJ proteins (claudin-1, -2, -3, -4, -5, -7, and -8) and AJ proteins (E-cadherin and β-catenin) in mucosal tissue samples obtained from the duodenum and colon of clinically normal dogs to provide baseline data about expression of these proteins in the canine duodenum and colon.

Materials and Methods

Sample—Biopsy specimens of duodenal and colonic mucosa were obtained endoscopically from 4 laboratory Beagles (3 sexually intact males and 1 sexually intact female). All dogs were 3 years old; body weight of the dogs ranged from 9.1 to 12.0 kg (median, 10.5 kg). None of the dogs had any clinical signs or evidence of weight loss for at least 1 year before the endoscopic procedures. Hematologic, serum biochemical, fecal, and urinalysis were performed on all dogs to assess health status. All experimental procedures were approved by the Laboratory Animal Experimentation Committee, Graduate School of Veterinary Medicine, Hokkaido University.

Tissue collection—Food was withheld from all dogs for 24 to 30 hours before the endoscopic procedures. On the day of endoscopy, dogs were sedated by administration of flunitrazepam (0.03 mg/kg, IV). Anesthesia was then induced by injection of thiopental (10 mg/kg, IV); anesthesia was maintained by administration of isoflurane in oxygen. All dogs received meloxicam (0.2 mg/kg, SC) as an analgesic before the endoscopic procedures.

Gastroduodenoscopy was performed in the anesthetized dogs by use of a flexible video endoscope. Multiple (8 to 10) mucosal biopsy specimens were collected from the descending duodenum and caudal duodenal flexure by use of nonserrated biopsy forceps. After gastroduodenoscopy was completed, colonoscopy was performed, and multiple (8 to 10) biopsy specimens were collected from the descending colon by use of the nonserrated biopsy forceps. During the endoscopic procedures, an ECG, respiratory rate, body temperature, arterial blood pressure, pulse oximetry, and capnography were monitored and recorded. All endoscopic procedures were completed within 2 hours, and all dogs recovered from anesthesia without complications.

Biopsy specimens for histologic examination were fixed in neutral-buffered 10% formalin, embedded in paraffin wax, and routinely processed for staining with H&E. Biopsy specimens for immunofluorescence microscopy and immunoblot analysis were snap-frozen in liquid nitrogen with and without optimal cutting temperature compound; respectively.

Immunoblot analysis—Two biopsy specimens of the duodenal mucosa or of the colonic mucosa from each dog were homogenized by use of a plastic pestle in 800 µL of lysis buffer that contained 50 mM Tris–HCl (pH, 7.4), 1 mM EDTA, 2% SDS, and a protease-inhibitor cocktail. Lysates were then incubated for 30 minutes on ice and passed through a biopolymer-shredding system. Protein concentration was determined via the Lowry method. Membranes were blocked with 5% nonfat milk, which was followed by incubation for 2 hours with the primary antibody. The following primary antibodies were used: rabbit polyclonal antibodies against claudin-1, -2, -3, -5, -7, and -8 (polyclonal antibody clone claudin-4; 0.23 µg/mL); anti-E-cadherin mouse monoclonal antibody (clone 3E2C1; 0.25 µg/mL); anti–E-cadherin mouse monoclonal antibody (clone 36/E-cadherin; 0.05 µg/mL); anti–β-catenin mouse monoclonal antibody (clone 14/β-catenin; 0.08 µg/mL); and anti–actin mouse monoclonal antibody (clone AC-74; 0.03 µg/mL). Membranes were then incubated for 1 hour with horseradish peroxidase–conjugated swine anti-rabbit IgG polyclonal antibody (0.11 µg/mL) or goat anti-mouse IgG polyclonal antibody (0.67 µg/mL). Signals were detected by use of a chemiluminescent detection reagent. Specificity of each of the primary antibodies was confirmed by the manufacturers, and specificity of each of the antibodies against claudin-1, -2, -3, and -4 was also confirmed by evaluation of the recombinant C-terminal peptide of each clau...
Without glutathione S-transferase, as described elsewhere.\textsuperscript{13}

**Immunofluorescence microscopy**—Seven-micrometer-thick frozen sections of duodenum and colonic mucosa were fixed by immersion in 95% ethanol at 4\textdegree{}C for 30 minutes followed by immersion in 100% acetone at 24\textdegree{}C for 1 minute. Sections then were incubated with 1% bovine serum albumin in PBS solution for 20 minutes. For single labeling, sections were incubated with the appropriate anti-claudin antibodies (1.25 \textmu{}g/mL for claudin-1, -2, -3, -5, and -8 and 2.5 \textmu{}g/mL for claudin-4 and -7; bovine serum albumin in PBS solution was used as the diluent), an anti–E-cadherin mouse monoclonal antibody (0.8 \textmu{}g/mL), or an anti–\textbeta{}-catenin mouse monoclonal antibody (0.8 \textmu{}g/mL) for 1 hour at 24\textdegree{}C. For dual labeling, sections were incubated with the anti–claudin-3 or -7 antibody and anti–ZO-1 mouse monoclonal antibody (clone ZO1-1A12; 2.5 \textmu{}g/mL)\textsuperscript{10} for 1 hour at 24\textdegree{}C. Sections incubated with normal rabbit or mouse IgG (2.5 \textmu{}g/mL) were included as a negative control sample. After incubation, sections were washed 3 times with PBS solution, which was followed by incubation for 30 minutes with goat anti-rabbit or goat anti-mouse IgG antibodies conjugated with fluorescent dyes\textsuperscript{10} (5 \textmu{}g/mL). After sections were washed with PBS solution, they were embedded in fluorescent mounting medium\textsuperscript{10} and examined by use of a fluorescence microscope equipped with a deconvolution apparatus.\textsuperscript{9}

**Results**

**Animals**—Each dog was judged to be healthy on the basis of results of physical examination, laboratory analyses, and abdominal ultrasonographic examination. Biopsy specimens of duodenal and colonic mucosa were histologically normal in all dogs.

**Expression of TJ and AJ proteins in canine duodenal and colonic mucosa**—Immunoblot analysis was performed to determine whether claudin-1, -2, -3, -4, -5, -7, and -8; E-cadherin; and \textbeta{}-catenin were expressed in the duodenal and colonic mucosa of the 4 dogs. Aliquots (5 \textmu{}g) of protein from each dog were analyzed, and similar amounts of \textbeta{}-actin were detected in all samples, which confirmed that they had equal loading (Figure 1). Because the sensitivity of each antibody was unknown, it was not possible to definitively determine the relative expression of TJ and AJ proteins within

![Figure 1](1048)

**Figure 1**—Photographs of immunoblotting results for expression of TJ and AJ proteins in biopsy specimens of duodenal and colonic mucosa obtained endoscopically from each of 4 clinically normal dogs (lanes 1 to 4). Values on the right side represent the apparent molecular mass (in kilodaltons) of each protein.

![Figure 2](1048)

**Figure 2**—Photomicrographs of representative immunofluorescence microscopy results for the distribution and localization of TJ and AJ proteins (claudin-3 [A and B], claudin-5 [C and D], claudin-7 [E and F], E-cadherin [G and H], \textbeta{}-catenin [I and J]) in the villi (left column) and epithelial crypts (right column) of duodenal and colonic mucosa obtained endoscopically from clinically normal dogs. A negative control specimen (stained with normal rabbit IgG) was included (K and L). The AJC region (arrows) and basolateral cell membrane (arrowheads) are indicated. M = Mucosal surface. Bars = 20 \textmu{}m.
the duodenum or colon. In the duodenal mucosa of all dogs, there was clear expression of claudin-3 and -5, E-cadherin, and β-catenin proteins. In addition, there was weak expression of claudin-7 protein and faint expression of claudin-1 protein in duodenal mucosa in all dogs. Expression of claudin-2, -4, and -8 proteins could not be detected in duodenal mucosa of any dog. In the colonic mucosa of all dogs, there was clear expression of claudin-2 and -3, E-cadherin, and β-catenin proteins. In addition, there was weak expression of claudin-5 and -7 proteins in the colonic mucosa of all dogs and faint expression of claudin-4 protein in the colonic mucosa of 1 dog. Expression of claudin-8 protein could not be detected in colonic mucosa of any dog. The anti–claudin-8 antibody used in the study reported here detected a single major band of approximately 20 kDa, as determined by use of immunoblotting with a tissue lysate derived from dog kidney (data not shown).

**Localization of TJ and AJ proteins in canine duodenal and colonic mucosa—**Expression and subcellular localization of claudin-1 -2, -3, -4, -5, -7, and -8; E-cadherin; and β-catenin in the duodenal and colonic mucosa of the 4 dogs was determined via immunofluorescence analysis. Representative images of sections of canine duodenum and colon labeled with anti-claudin, anti-E-cadherin, and anti–β-catenin antibodies were examined (Figures 2 and 3). In the duodenal and colonic mucosa, claudin-3–specific, E-cadherin–specific, and β-catenin–specific labeling was most intense at the epithelial cell AJC, with fainter labeling along the basolateral membrane. Although staining for claudin-5 protein was weak in the duodenal and colonic mucosa, compared with staining for other TJ and AJ proteins, claudin-5 labeling was most intense at the AJC with weak labeling along the basolateral membrane. In contrast, claudin-7 protein was expressed primarily at the basolateral membrane. Similar to staining for claudin-5, staining for claudin-7 was weak, compared with staining for other TJ and AJ proteins. These claudins and E-cadherin were uniformly expressed along the length of the crypt-to-villi axis of the duodenal mucosa and crypt-to-luminal surface axis of the colonic mucosa obtained from the clinically normal dogs. In contrast, expression of β-catenin protein was slightly weak in the crypts of duodenal and colonic mucosa, and the differences in the staining of β-catenin between the epithelial cell AJC and basolateral membrane were more pronounced in the crypt region. Claudin-2–specific labeling was also detected at the epithelial cell AJC of colonic mucosa, whereas expression of claudin-2 protein decreased from the crypt to the luminal surface. Immunofluorescent labeling with 2 dyes revealed that claudin-3 and ZO-1 were colocalized at the apical intercellular junction of the duodenal mucosal epithelium but that claudin-7 was not colocalized with ZO-1 (Figure 4). These immunofluorescent labeling patterns for claudin-3 and -7 with ZO-1 were also observed in the colonic mucosa (data not shown). Labeling for claudin-1, -4, and -8 labeling was not observed in the duodenal and colonic mucosa of any dog. The anti–claudin-1, -4, and -8 antibodies used in the study reported here stained the APC for these claudins in the glomeruli and renal tubules of dog kidney (data not shown).

**Discussion**

In the study reported here, expression and localization of a number of key TJ and AJ proteins in the duodenum and colon of clinically normal dogs was elucidated. This led to several important conclusions.

Analysis of results of the immunoblot and immunofluorescence analyses indicated that expression of claudins in the canine duodenum and colon differed from that reported for human and rat intestines. In humans, immunoblot analysis has revealed expression

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**Figure 3**—Photomicrographs of representative immunofluorescence microscopy results for the distribution and localization of TJ and AJ proteins (claudin-2 [A and B], claudin-3 [C and D], claudin-5 [E and F], claudin-7 [G and H], E-cadherin [I and J], and β-catenin [K and L]) in the luminal surface (left column) and epithelial crypts (right column) of colonic mucosa obtained endoscopically from clinically normal dogs. S = Luminal surface. Bars = 20 µm. See Figure 2 for remainder of key.
of claudin-1, -3, -4, -5, and -7 proteins and claudin-1, -3, -4, -5, -7, and -8 proteins in duodenal and colonic mucosa, respectively. In addition, investigators in another study reported expression of claudin-2, -3, -4, and -5 proteins in rat duodenum and colon by use of immunoblotting and immunofluorescence microscopy. In canine duodenal mucosa, claudin-1 expression was extremely low, which may explain the difficulty in detecting an immunofluorescent signal of claudin-1 in canine duodenum. Claudin-4 was not detected during immunoblotting. In addition, canine colonic mucosa lacked claudin-1, -4, and -8, although colonic mucosa from 1 dog had a faint signal for claudin-4 during immunoblotting. Claudin-1, -4, and -8 can reduce paracellular permeability to cations, and canine duodenal and colonic mucosa might be more permeable to cations than are human and rat duodenal and colonic mucosa. Claudin-2 was not detected in canine duodenum, but it was found to be clearly expressed in canine colon, as determined by use of immunoblotting and immunofluorescence microscopy. Claudin-5 was clearly expressed in the duodenum but was only weakly expressed in the colon, as determined via immunoblotting. In contrast, there appeared to be no clear difference in the intensity of immunofluorescence signals for claudin-5 between the duodenum and colon. The reason for this discrepancy in the present study is not clear, but immunoblotting analysis is a more quantitative method than is immunofluorescence staining, and immunoblotting is suitable for comparing protein expression. Claudin-2 likely acts as a paracellular pore to cations. In contrast, claudin-5 reduces paracellular permeability to cations in Madin-Darby canine kidney cell monolayers. The relative abundance of these claudins may contribute to the charge selectivities in the duodenum and colon. For example, potassium ions are absorbed primarily in the colon through a paracellular pathway, and claudin-2 plays a role in absorption of these ions. In a study in humans with IBD, investigators identified increased expression of claudin-2 and decreased expression of claudin-5 in colonic mucosa obtained from patients with Crohn’s disease. In addition, investigators in another study reported that immunofluorescent staining for claudin-2 was markedly increased in the crypt and luminal surface of colonic mucosa from dogs with LPC, compared with results for colonic mucosa obtained from clinically normal dogs in which claudin-2 was barely detectable at the luminal surface. These changes in expression of claudins may cause the colonic epithelial monolayer to become more permeable; thus, they may play a role in the pathophysiology of IBD in dogs and humans. It is also considered important to evaluate changes in the distribution of TJ and AJ proteins along the villus-to-crypt axis of the duodenum and luminal surface-to-crypt axis of the colon in afflicted patients, although there are limitations for interpretation of endoscopically obtained mucosal biopsy specimens with respect to orientation of specimens and crush artifacts.

In the study reported here, we found that claudin-7 was expressed exclusively at the basolateral membrane. Although staining for claudin-7 was extremely weak, compared with the immunofluorescent signals of other TJ and AJ proteins, staining for claudin-7 was not observed in control specimens. Therefore, we concluded that staining for claudin-7 was a specific signal. Faint staining for claudin-7 may have reflected low-level expression in the canine duodenum and colon or low sensitivity of the anti–claudin-7 antibody that was used for immunofluorescent staining, compared with the sensitivity for immunoblotting. These explanations may also be true of staining for claudin-5. There are many reports that claudins can localize at the basolateral membrane instead of, or in addition to, localization at the TJ. Although the role of claudins localized at the basolateral membrane remains obscure, they may simply represent a storage pool that can be recruited to the AJC when needed. Alternatively, claudins localized at the basolateral membrane may have some other novel function, such as participating in cell-cell or cell-matrix adhesion or signaling. In a study conducted to determine associations with disease, there was redistribution of claudin-5 to the basolateral membrane in the colonic mucosa of patients with Crohn’s disease, whereas claudin-5 was strictly localized at the TJ in the colonic mucosa of healthy control humans. Therefore, it is important to examine the subcellular localization of claudins in the intestinal mucosa from dogs with IBD.

With regard to expression of AJ proteins, the membranous localization of E-cadherin and β-catenin in canine duodenum and colon was investigated in the study reported here. Our results were in agreement with those in other studies conducted on their expression in human duodenum and colon and canine colon. A reduction in E-cadherin expression has been detected in intestinal mucosal epithelium obtained at the site of

Figure 4—Photomicrographs of representative dual-immunofluorescent microscopy results for biopsy specimens of duodenal mucosa obtained endoscopically from clinically normal dogs. The upper row represents the dual-immunostaining patterns for claudin-3 (A) and ZO-1 (B) and the merged image for claudin-3 and ZO-1 (C). The bottom row represents the dual-immunostaining patterns for claudin-7 (D) and ZO-1 (E) and the merged image for claudin-7 and ZO-1 (F). Claudin-3 is colocalized with ZO-1 at the apical TJ region, whereas claudin-7 is localized at the basolateral membrane. Bars = 20 µm.
ulcers in humans with IBD. In contrast, there were no changes in immunofluorescent signals for E-cadherin in colonic mucosa obtained from dogs with LPC. One reason for this is that the findings in that study may reflect only the qualitative nature of E-cadherin expression; therefore, further evaluation with a more quantitative method, such as immunoblotting, will be necessary to reveal changes in E-cadherin expression in the intestinal mucosa of dogs with IBD.

We concluded that the study reported here has provided important initial data on the expression and distribution of a number of important TJ and AJ proteins in the canine duodenum and colon. Our findings should be of value in the evaluation of the contributions of TJ and AJ proteins to IBD in dogs.

References