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Gene expression in intestinal mucosal biopsy specimens obtained from dogs with chronic enteropathy

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Objective—To characterize mucosal gene expression in dogs with chronic enteropathy (CE).

Animals—18 dogs with CE and 6 healthy control dogs.

Procedures—Small intestinal mucosal biopsy specimens were endoscopically obtained from dogs. Disease severity in dogs with CE was determined via inflammatory bowel index scores and histologic grading of biopsy specimens. Total RNA was extracted from biopsy specimens and microchip array analysis (approx 43,000 probe sets) and quantitative reverse transcriptase PCR assays were performed.

Results—1,875 genes were differentially expressed between dogs with CE and healthy control dogs; 1,582 (85%) genes were downregulated in dogs with CE, including neurotensin, fatty acid-binding protein 6, fatty acid synthase, aldehyde dehydrogenase 1 family member B1, metallothionein, and claudin 8, whereas few genes were upregulated in dogs with CE, including genes encoding products involved in extracellular matrix degradation (matrix metalloproteinases 1, 3, and 13), inflammation (tumor necrosis factor, interleukin-8, peroxisome proliferator-activated receptor γ , and S100 calcium-binding protein G), iron transport (solute carrier family 40 member 1), and immunity (CD96 and carcinoembryonic antigen-related cell adhesion molecule [CEACAM] 18). Dogs with CE and protein-losing enteropathy had the greatest number of differentially expressed genes. Results of quantitative reverse transcriptase PCR assay for select genes were similar to those for microchip array analysis.

Conclusions and Clinical Relevance—Expression of genes encoding products regulating mucosal inflammation was altered in dogs with CE and varied with disease severity.

Impact for Human Medicine—Molecular pathogenesis of CE in dogs may be similar to that in humans with inflammatory bowel disease. (*Am J Vet Res* 2012;73:1219–1229)

Chronic enteropathies are a group of disorders that cause gastrointestinal tract inflammation and persistent or recurrent signs of gastrointestinal tract dis-

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ABBREVIATIONS

CD	Crohn's disease
CE	Chronic enteropathy
CIBDAI	Canine inflammatory bowel disease activity index
GO	Gene ontology
IBD	Inflammatory bowel disease
NF- κ B	Nuclear factor- κ B
PLE	Protein-losing enteropathy
QRT-PCR	Quantitative reverse transcriptase PCR
UC	Ulcerative colitis

ease in dogs. The most common causes include adverse gastrointestinal tract reaction to food, idiopathic IBD, and antimicrobial-responsive enteropathy.¹⁻⁴ The pathogenesis of CE in dogs likely involves several factors including host genetics, intestinal microenvironment (bacteria and diet), immune system function, and environmental triggers of intestinal inflammation.^{1,5-7} Strategies for diagnosis of CE include extensive evalua-

tion of patients to detect underlying diseases and histologic evaluation of intestinal mucosal biopsy specimens for detection of lymphocytic-plasmacytic, eosinophilic, or granulomatous inflammation.^{1,2}

Dysregulated gene expression is important in the pathogenesis of CEs in humans, including IBD.⁸ Various genes encoding for proteins involved in fatty acid absorption and synthesis have different levels of expression in the ileum and colon of humans with CD and UC.⁹ Results of another study¹⁰ in which microchip array analysis of mRNA from mucosal biopsy specimens was performed indicate genes encoding for enzymes responsible for detoxification (eg, cytochrome P450) are downregulated in cells of the colonic mucosa of humans with IBD. Genes encoding for chemokines, markers of neutrophil activation, and anti-inflammatory factors are upregulated in humans with CE.^{11,12} Moreover, disturbances in expression of genes encoding for proteins involved in innate or adaptive immunity, detoxification, and nuclear transcription are important in the pathogenesis of mucosal inflammation in mice with experimentally induced colitis.^{13,14}

The objectives of the study reported here were to determine gastrointestinal mucosal gene expression patterns in dogs with CE, compare results with those for healthy control dogs, and determine whether gene expression differs among dogs with CE of various severities. In addition, expression of genes in dogs with CE was compared with that reported for humans with IBD.

Materials and Methods

Animals—Eighteen dogs with CE were included in the study. Criteria for selection of dogs with CE included a history of chronic vomiting, diarrhea, or weight loss of at least 6 weeks' duration, exclusion of identifiable underlying disorders (via performance of a thorough laboratory diagnostic evaluation), and histopathologic evidence of gastrointestinal mucosal inflammation consistent with a diagnosis of idiopathic CE. Prior to enrollment, dogs had been fed an elimination diet exclusively for 3 weeks followed by treatment with metronidazole (10 mg/kg, PO, q 12 h for 3 weeks); dogs selected for the study had failed to improve with either treatment. All medications were discontinued at least 2 weeks before collection of gastrointestinal mucosal specimens. Disease severity was determined by use of the CIBDAI,¹⁵ which included assessment of the following 6 variables: attitude and activity, appetite, vomiting, fecal consistency, fecal frequency, and weight loss. After summation of scores for each of the 6 variables, a total composite score was determined, which indicated clinically unimportant (score, 0 to 3), mild (score, 4 to 5), moderate (score, 6 to 8), or severe (score, 9 or greater) clinical signs of CE.

Six mixed-breed dogs owned by Iowa State University were included in the healthy control group. Control dogs were judged to be healthy on the basis of unremarkable physical examination, CBC, serum biochemical analysis, urinalysis, and direct or indirect fecal evaluation (for detection of parasites) results. None of the healthy control dogs had signs of gastrointestinal tract disease during the 6 weeks prior to the start of the study.

Approval to conduct the study was obtained from the Iowa State University Institutional Animal Care and Use Committee. Owners of dogs with CE provided informed written consent prior to enrollment of those dogs in the study.

Collection of tissue biopsy specimens—Food was withheld from dogs for 12 hours prior to collection of tissue specimens. Mucosal biopsy specimens were endoscopically obtained from the stomach and duodenum of each dog for histologic examination as part of a standard esophagoduodenoscopy protocol performed at Iowa State University. Gastric biopsy specimens were obtained for microscopic evaluation to detect gastric mucosal inflammation attributable to causes other than CE (eg, *Helicobacter* spp-associated gastritis). Each dog underwent a standard anesthetic protocol; butorphanol was administered IV as a preanesthetic agent, propofol was administered IV as an induction agent, and isoflurane (administered at a concentration of 2% to 3% in oxygen delivered with an inhalation gas delivery system) was used to maintain anesthesia. Ten to 12 mucosal biopsy specimens were obtained from the proximal to middle portions of the duodenum of each dog. Four mucosal biopsy specimens were obtained from each colonic segment (ascending, transverse, and descending) of 4 dogs that underwent colonoscopy. Colonoscopy was performed in these 4 dogs because they had clinical signs of enterocolitis with inflammation of both the small and large intestines. Three duodenal mucosal biopsy specimens from each dog were placed in a commercial liquid RNA preservative^a immediately after collection and kept at -80°C until isolation of RNA. The remaining specimens were placed in 10% neutral-buffered formalin, routinely processed, and stained with H&E for histologic evaluation. Sections of intestinal mucosal biopsy specimens were scored by use of standardized criteria to determine type and amount of inflammatory cell infiltrate and architectural changes in tissue.¹⁶ All intestinal mucosal biopsy specimen sections were evaluated by a board-certified veterinary pathologist (MRA) with experience in gastrointestinal pathology.

RNA extraction and cDNA synthesis—Total RNA was extracted from 2 intestinal mucosal biopsy specimens of each dog by use of a commercial RNA extraction kit^b in accordance with the manufacturer's protocol for extraction of RNA from animal tissue. Purity and integrity of RNA were assessed via a bioanalyzer with a reagent set.^c Control samples that did not undergo reverse transcription and had nuclease-free water instead of RNA were prepared. First-strand cDNA was synthesized by use of random hexamers and oligo(dT) primers^d in accordance with the manufacturer's instructions. None of the RNA samples had evidence of genomic DNA after amplification.

Microchip array analysis—Total RNA was transported to the University of Minnesota Biomedical Genomics Center Microarray Facility for labeling, hybridization, and acquisition of images. Microchip array analysis was performed with an array platform.^e Control samples included 4 spiked control samples for

labeling (lys, phe, thr, and dap genes of *Bacillus subtilis*), 4 exogenous spiked control samples for hybridization (bioB, bioC, and bioD genes of *E coli* and the cre gene of the P1 bacteriophage), and the housekeeping genes glyceraldehyde 3-phosphate dehydrogenase and β -actin for normalization of gene expression data. The order in which samples were prepared was determined by use of a computer-generated randomization procedure prior to processing to avoid systematic bias. Hybridization reactions were performed with reagents^f in accordance with the manufacturer's instructions and as previously described.¹⁷ After hybridization, microchips were washed and stained with a streptavidin-conjugated phycoerythrin dye enhanced with biotinylated goat anti-streptavidin antibody via a wash and stain station^g and associated software. Images of microchips were acquired by use of a scanning system.^h

The microchip array^c included 43,036 probes to simultaneously detect approximately 18,000 *Canis familiaris* mRNA or expressed sequence tag-based transcripts and > 20,000 nonredundant predicted genes. Sequences in the microchip array were obtained from *C familiaris* UniGene build No. 11 (April 2005); GenBank mRNA sequences up to April 15, 2005; and gene predictionsⁱ from the Boxer genome.^j Each RNA sample from each dog was treated as an individual unit for analysis.

QRT-PCR assay—To confirm results of microchip array analysis, some of the genes with > 3.0-fold differences in expression among dogs with CE of various severities were assayed with asymmetric cyanine dye-based 2-step real-time QRT-PCR assay (Appendix); cDNA prepared from each total RNA sample was used. Gene-specific primer sets were designed by use of software,^k and at least one of the primers in each primer pair spanned a genomic intron. Target sequences in cDNA samples were detected with QRT-PCR assay by use of a dye mixture.^l For normalization of data, expression of the canine β -glucuronidase gene was used as the reference because it had stable expression in intestinal mucosal biopsy specimens obtained from dogs of all groups.

Prior to analysis of cDNA samples, all QRT-PCR assay gene targets were evaluated on a test plate via serial dilutions (range, undiluted to 1:100,000) of a mixture consisting of an equal portion of each of the 24 (6 control and 18 CE dogs) study cDNA samples. Results of this test plate QRT-PCR assay indicated the cDNA sample dilution range within which quantitative PCR inhibition was absent and within which standard curves for all targets could be reliably generated. Calculation of results was performed via a spreadsheet software^m-based method.¹⁸ Test plate QRT-PCR assay results indicated that the cDNA samples had no quantitative PCR-inhibitory properties, and a common dilution range (1:5 to 1:250 [1.319 to 0.0263 ng of cDNA/ μ L]) for all target sequence standard curves was determined by use of the spreadsheet software^m-based method.¹⁸

Standard curves with 5 data points were generated for each target sequence within the common dilution range. Each cDNA sample was diluted to a concentration of approximately 2.8637 ng/ μ L before performance of QRT-PCR assays so that addition of 6 μ L of cDNA

to each 25- μ L assay mixture resulted in a final cDNA concentration of 0.6873 ng/ μ L. This concentration value had been calculated via the spreadsheet software^m-based method as the mean of the first 2 points of each target sequence standard curve. The reaction mixture also contained forward and reverse primers (500nM each; Appendix), nuclease-free water, 3mM MgCl₂, and 500nM (final concentration) free passive reference dye.ⁿ The cDNA samples, control samples without template sequences, and standard curve samples were placed in duplicate in wells of 96-well QRT-PCR assay plates. Plates were placed in a sequence detection system^o and exposed to the following thermoprotocol: 2 minutes at 50°C, 2 minutes at 95°C, 40 cycles of 15 seconds at 95°C, and 30 seconds at 60°C, then melt curve analysis at 60°C. Cycle values were determined at an appropriate threshold for each target sequence by use of software,^o and relative quantitative analysis was performed manually by use of custom spreadsheet files.^m Sample signals determined to have resulted from primer-dimer formation (on the basis of evaluation of melt curves) were excluded from data analysis. Cycle values for each target sequence in each cDNA sample were interpreted by use of the standard curve for each of those target sequences. The resulting relative target sequence expression values were normalized relative to β -glucuronidase expression. Relative expression data were calculated by use of an efficiency-corrected method.¹⁹

Statistical analysis—Data from the 24 (6 control and 18 CE dogs) microchip array analyses were normalized by use of the robust multiarray mean normalization method.²⁰ For each of the approximately 43,000 probe sets on the microchip array, a standard permutation test was applied to the normalized data to test for differences in distribution of target sequence expression between healthy control dogs and dogs with CE. Permutation tests yielded a *P* value for each of the probe sets. These *P* values were converted to *q* values as previously described.²¹ Genes that had different expression between groups of dogs and an estimated false discovery rate of 1% (*q* < 0.01) were identified. Only results for probe sets having an estimated \geq 2-fold difference in expression between groups of dogs were evaluated further.

A Fisher exact test was used to identify GO terms that were overrepresented among the probe sets for genes that were determined to have different expression between groups of dogs. One test for overrepresentation was conducted for each of the GO terms that was associated with probe sets with statistically significant results; GO annotation information was provided by the microarray chip manufacturer.^p Functional attributions of genes were determined via a database^q with the default parameters.

To determine whether gene expression was associated with severity of clinical disease, microchip array analysis results for 17 genes that had > 3.0-fold differences in expression among dogs with CE of various severities (on the basis of CIBDAI values) were statistically compared. These genes included chemokine ligand 13, aldehyde dehydrogenase 1 family member B1, metallothionein, tachykinin precursor 1, solute car-

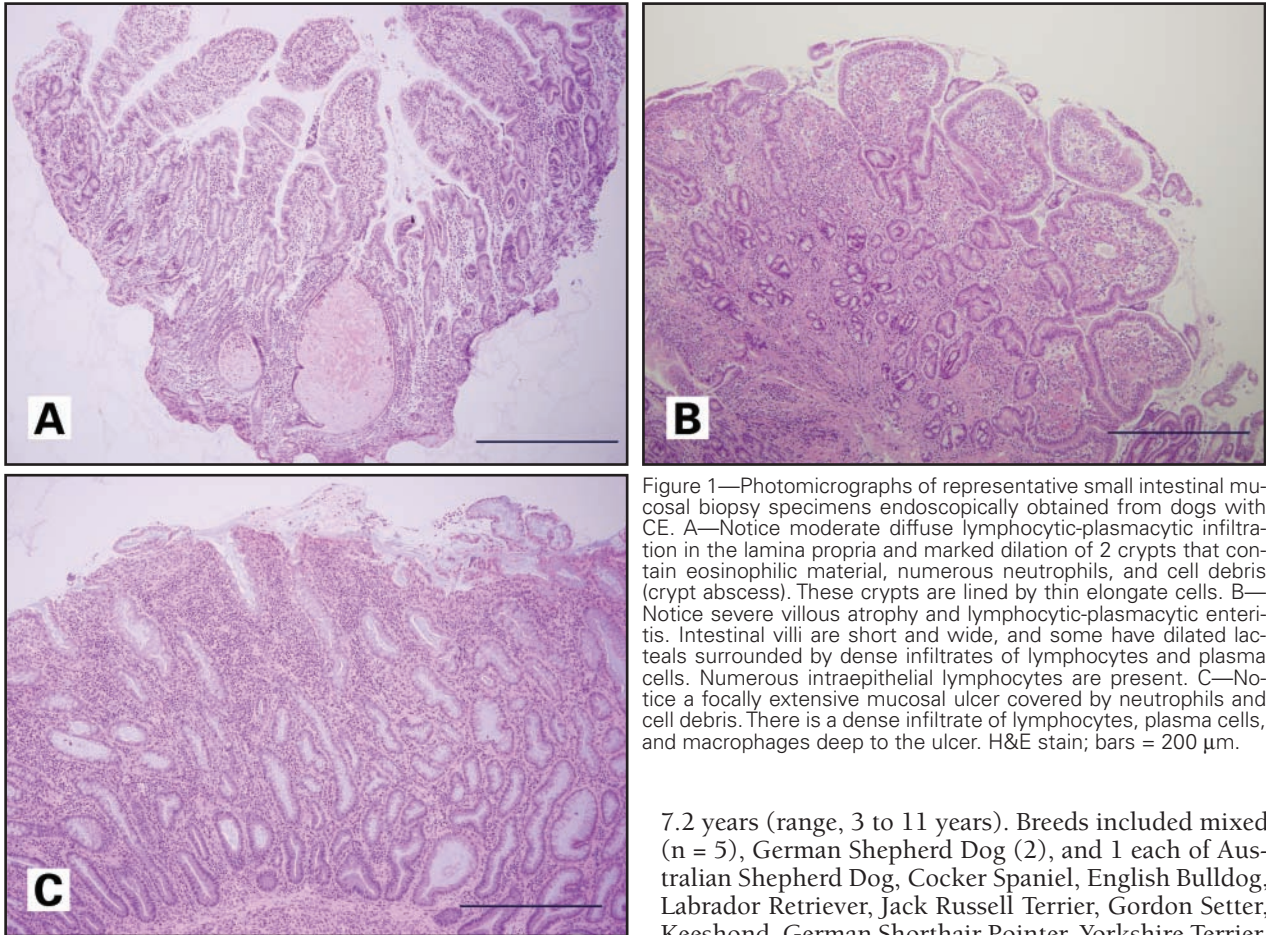


Figure 1—Photomicrographs of representative small intestinal mucosal biopsy specimens endoscopically obtained from dogs with CE. A—Notice moderate diffuse lymphocytic-plasmacytic infiltration in the lamina propria and marked dilation of 2 crypts that contain eosinophilic material, numerous neutrophils, and cell debris (crypt abscess). These crypts are lined by thin elongate cells. B—Notice severe villous atrophy and lymphocytic-plasmacytic enteritis. Intestinal villi are short and wide, and some have dilated lacteals surrounded by dense infiltrates of lymphocytes and plasma cells. Numerous intraepithelial lymphocytes are present. C—Notice a focally extensive mucosal ulcer covered by neutrophils and cell debris. There is a dense infiltrate of lymphocytes, plasma cells, and macrophages deep to the ulcer. H&E stain; bars = 200 μ m.

rier family 40 member 1, RNase A family 1, caspase 3, CD96 molecule, granzyme B, matrix metalloproteinase 1, fatty acid-binding protein 6, glucagon, claudin 8, S100 calcium-binding protein G, cell division cycle associated 3, peroxisome proliferator-activated receptor γ , and neurotensin. An ANOVA overall *F* test was used to identify significant differences among results for dogs with various severities of CE (dogs with moderate CE, dogs with severe CE but not PLE, dogs with PLE) and healthy control dogs; this was intended to identify associations between gene expression and disease severity. This analysis was performed separately for each gene. Then, pairwise comparisons were performed between gene expression values for each group of dogs for genes with an overall *F* test value that was significant ($P < 0.05$).

Gene expression in mucosal biopsy specimens obtained from dogs with CE in the present study determined with microchip array analysis was compared with expression of genes in humans with IBD determined in other studies. Results of gene expression profiles in humans with CD or UC were obtained via literature searches for manuscripts published from 1998 through 2010.

Results

Demographics and clinical characteristics of dogs—Of the 18 dogs with CE, 8 were males (3 sexually intact and 5 neutered) and 10 were females (1 sexually intact and 9 neutered). Mean age of these dogs was

7.2 years (range, 3 to 11 years). Breeds included mixed ($n = 5$), German Shepherd Dog (2), and 1 each of Australian Shepherd Dog, Cocker Spaniel, English Bulldog, Labrador Retriever, Jack Russell Terrier, Gordon Setter, Keeshond, German Shorthair Pointer, Yorkshire Terrier, Shih Tzu, and Beagle. All dogs with CE had moderate ($n = 10$) to severe (8) disease; mean CIBDAI score was 7.8 (range, 6 to 12). Five of the 8 dogs with severe CE had panhypoproteinemia (mean serum albumin concentration, 1.7 g/dL [range, 1.2 to 1.9 g/dL]; mean total protein concentration, 3.5 g/dL [range, 3.0 to 3.9 g/dL]), which was consistent with a diagnosis of PLE; these dogs had a mean CIBDAI score of 8.6. Abnormalities of duodenal mucosa (ie, increased granularity, friability, or erosions) were endoscopically observed in all 18 dogs with CE. Four of these dogs also underwent colonoscopy; each of these dogs had colonic erosions and areas of colonic mucosa that were friable. Histologic examination of intestinal mucosal biopsy specimens indicated 8 dogs had mild and 10 dogs had moderate to severe inflammatory lesions (Figure 1).

Mean age of the 6 healthy control dogs was 4.3 years (range, 3 to 6 years). Results of laboratory tests, endoscopic examinations, and histologic examination of mucosal biopsy specimens obtained from these control dogs were unremarkable.

Gene expression determined via microchip array—Target gene expression was significantly different between healthy control dogs and dogs with CE for 1,875 of the probe sets. Healthy control dogs and dogs with CE had significantly different expression of genes associated with protein synthesis and transport, cell replication, intracellular organelles, and extracellular matrix degradation (data not shown). Selective filter-

Table 1—Expression of select genes in intestinal mucosal biopsy specimens endoscopically obtained from 18 dogs with CE as determined with microchip array analysis.

Gene name	Abbreviation	Function of protein	Fold difference in expression*
RNase A family 1	Ribonucleic acid SE1	RNA hydrolysis	57
Matrix metalloproteinase 1	MMP1	Extracellular matrix degradation	11
S100 calcium-binding protein G	S100G	Calcium transport and activity	11
Solute carrier family 40 member 1	SLC40A	Iron transport	11
Peroxisome proliferator-activated receptor γ	PPARG	Regulates NF- κ B pathway	4
Tumor necrosis factor	TNF	Promotes inflammation	3
Interleukin-8	IL-8	Induces chemotaxis	3
CD96 molecule	CD96	Adaptive and innate immunity	3
Caspase 3	CASP3	Cellular apoptosis	3
Granzyme B	GZMB	Cytotoxic T lymphocyte-mediated apoptosis	3
Neurotensin	NTS	Neuropeptide signaling	-92
Glucagon	GCG	Counters insulin, cell proliferation	-67
Aldehyde dehydrogenase 1 family member B1	ALDH1B1	Metabolizes alcohol	-16
Regenerating islet-derived 3 gamma	REG3G	Promotes inflammation	-8
Fatty acid-binding protein 6	FABP6	Fatty acid uptake and transport	-13
Tachykinin precursor 1	TAC1	Gastrointestinal tract neuropeptide	-8
Metallothionein	MTIE	Barrier function	-7
Claudin 8	CLDN8	Tight junction protein	-6
Chemokine ligand 13	CXCL13	B-cell chemoattractant	-4
Cell division cycle associated 3	Centers for Disease Control and Prevention A3	Promotes mitosis	-4
Fatty acid synthase	FASN	Fatty acid synthesis	-3

*Results are expressed relative to expression of genes in intestinal mucosal biopsy specimens endoscopically obtained from 6 healthy control dogs. Expression of all genes listed is significantly ($P < 0.05$) different between dogs with CE and healthy control dogs.

Table 2—Results of pairwise comparisons of gene expression in intestinal mucosal biopsy specimens endoscopically obtained from 18 dogs with CE of various severities and 6 healthy control dogs.

Gene	Abbreviation	Pairwise comparison			
		PC	PS	PM	SC
Chemokine ligand 13	CXCL13	< 0.001*	0.03*	0.04*	0.04*
Aldehyde dehydrogenase 1 family member B1	ALDH1B1	< 0.001*	0.03*	0.05	0.10
Metallothionein	MTIE	< 0.001*	0.03*	0.04*	0.08
Tachykinin precursor 1	TAC1	< 0.001*	0.03*	0.03*	0.07
Solute carrier family 40 member 1	SLC40A	< 0.001*	0.03*	0.04*	0.11
RNase A family 1	Ribonucleic acid SE1	< 0.001*	0.03*	0.04*	0.09
Caspase 3	CAP3	0.002*	0.11	0.16	0.24
CD96 molecule	CD96	0.008*	0.27	0.10	0.19
Granzyme B	GZMB	0.03*	0.56	0.08	0.20
Matrix metalloproteinase 1	MMP1	0.005*	0.03*	0.06	0.73
Fatty acid-binding protein 6	FABP6	< 0.001*	0.07	0.25	0.09

Data are P values for pairwise comparisons of expression of genes. Results for 17 genes with > 3.0-fold different expression among dogs with CE of various severities (moderate CE, severe CE but not PLE, and PLE; determined by use of the CIBDAI¹⁵) were compared. Genes with significantly ($P < 0.05$) different expression among dogs with various severities of CE and healthy control dogs were selected for further analysis. Expression of these genes was compared in a pairwise manner between groups of dogs.

*Expression of gene is significantly ($P < 0.05$) different between the groups of dogs for which results are compared in the column.

PC = Dogs with PLE versus healthy control dogs. PM = Dogs with PLE versus dogs with moderate CE. PS = Dogs with PLE versus dogs with severe CE but not PLE. SC = Dogs with severe CE but not PLE versus healthy control dogs.

ing (fold enrichment ≥ 1.3 and false discovery rate < 1%) of genes indicated that expression of most differentially expressed genes was lower in intestinal mucosal biopsy specimens obtained from dogs with CE versus those obtained from healthy control dogs. Of the 1,875 genes, expression of 1,582 (84%) was lower in intestinal mucosal biopsy specimens obtained from dogs with CE versus those obtained from healthy control dogs, including genes with annotations indicating associations with mucosal homeostasis, mitosis and cell cycle, and

cell-to-cell signaling. Few genes had significantly higher expression in mucosal biopsy specimens obtained from dogs with CE versus those obtained from control dogs; these included genes encoding for the matrix metalloproteinases and proteins associated with inflammation and immune function.

Results for select genes with significantly different expression between dogs with CE and healthy control dogs were summarized (Table 1). Neurotensin was the most highly downregulated (compared with expression

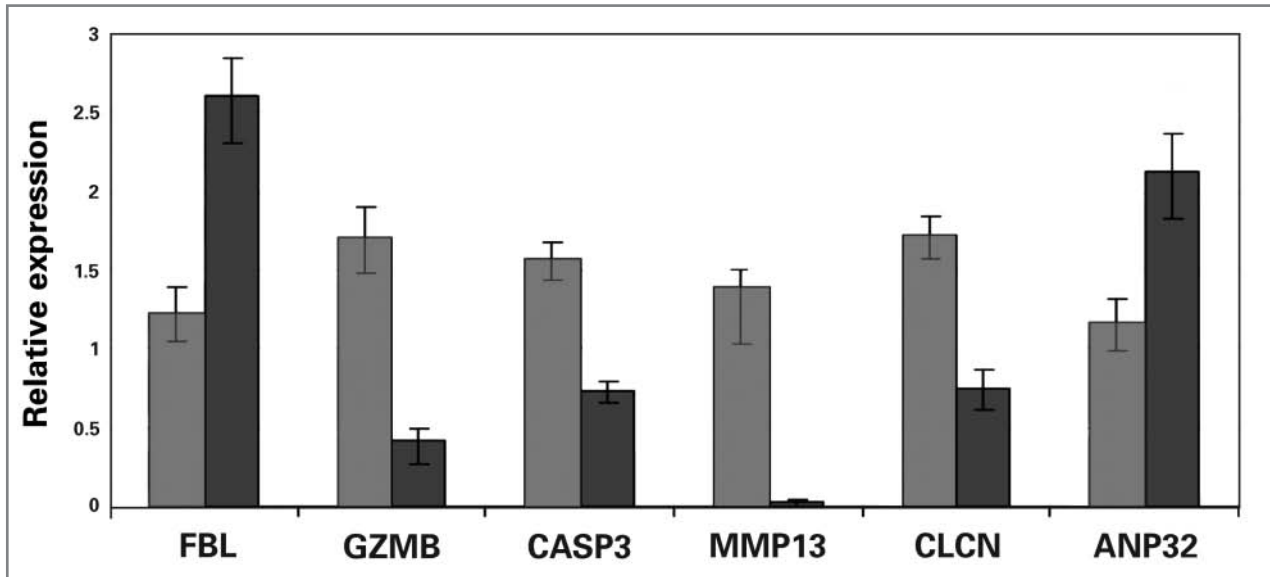


Figure 2—Mean ± SEM expression of select genes in intestinal mucosal biopsy samples endoscopically obtained from 18 dogs with CE (light gray bars) and 6 healthy control dogs (dark gray bars). Values were determined via QRT-PCR assay. Expression values are normalized relative to expression of β glucuronidase. Expression of each of the genes is significantly ($P < 0.05$) different between groups of dogs. ANP32 = ANP32 family member A. CASP3 = Caspase 3. CLCN = Chloride channel 3. FBL = Fibrillarin. GZMB = Granzyme B. MMP1 = Matrix metalloproteinase 1.

Table 3—Comparison of gene expression in dogs with CE in the present study versus that in humans with IBD* reported by other authors.

Gene function and abbreviation	Name of gene	Type of IBD	References	Relative gene expression in dogs with CE†
Immune and inflammatory response				
CD96	CD96 molecule	CD ^a	63	3
IL-8	Interleukin-8	CD and UC	8 and 63	3
TNF	Tumor necrosis factor	CD ^a and UC ^c	60 and 62	3
CXCL13	CXCL13	—	—	-4
PPARG	Peroxisome proliferator-activated receptor γ	CD ^a	63	4
Cell proliferation and growth				
REG3G	Regenerating islet-derived 3 gamma	UC	8 and 60	-8
Ribonucleic acid SE1	RNase family genes	UC	8	57
GCG	Glucagon	CD	8 and 63	-67
Centers for Disease Control and Prevention A3	Cell division cycle associated protein 3	—	—	-4
S100G	S100 calcium-binding protein G	CD and UC	8 and 63	11
CASP3	Caspase 3	—	—	3
GZMB	Granzyme B	CD ^c	61	3
Cell structure and permeability				
MT1E	Metallothionein 1E	UC	8	-7
MT2A	Metallothionein 2A	CD ^{a,c}	63	-5
MMP1	Matrix metalloproteinase 1	CD and UC	8 and 63	11
MMP3	Matrix metalloproteinase 3	CD and UC	8 and 60	3
MMP13	Matrix metalloproteinase 13	UC ^{b,c}	64	4
CLDN8	Claudin 8	CD ^a	58	-6
Metabolic pathways and transport				
SLC40A	Solute carrier family 40 member 1	CD ^a	54	11
ALDH1B1	Aldehyde dehydrogenase 1 family member B1	CD ^a	58 and 63	-16
FABP6	Fatty acid-binding protein 6	CD and UC	9 and 58	-13
—	Fatty acid synthase	CD and UC	9	-3
CPO	Carboxypeptidase	CD ^a	58	-7
Neuropeptide signaling				
NTS	Neurotensin	CD ^a	58	-92
TAC1	Tachykinin precursor 1	—	—	-8

Data are comparisons of mRNA expression values unless otherwise indicated.

*Genes with increased or decreased expression in humans with CD or UC. †Results are expression of genes in dogs with CE relative to expression in humans with IBD.

— = Not applicable.

^aGene expression results only for humans with CD. ^bGene expression results only for humans with UC. ^cResults are for protein expression.

in healthy control dogs) gene in dogs with CE (expression 92-fold as low). Other genes significantly downregulated in dogs with CE included glucagon (expression 67-fold as low), aldehyde dehydrogenase 1 family member B1 (18-fold as low), and fatty acid-binding protein 6 (15-fold as low). Both metallothionein 1E and metallothionein 2A genes were downregulated (expression 7- and 5-fold as low, respectively) in CE dogs, compared with gene expression in control dogs. Genes with significantly higher expression in dogs with CE versus healthy control dogs included RNase A family 1 (expression 57-fold as high) and matrix metalloproteinase 1, solute carrier family 40 member 1, and the S100 calcium-binding protein family of genes including S100G, S100A2, and S100A10 (11-, 2-, and 2-fold as high, respectively).

Gene expression in dogs with various severities of CE—Seventeen genes were > 3.0-fold differentially expressed (as determined with microchip array analysis) among healthy control dogs and dogs with moderate CE, severe CE but not PLE, and PLE. Of these 17 genes, expression for 11 was significantly ($P < 0.02$) different among the 4 groups of dogs. Significant differences in expression were detected among dogs with PLE and those with moderate or severe CE on the basis of CIBDAI scores (Table 2). Dogs with PLE had the highest CIBDAI scores; these dogs had the highest number of genes with significantly different expression in mucosal biopsy specimens, compared with that in healthy control dogs ($n = 11$ genes), dogs with severe CE but not PLE (7), and dogs with moderate CE (5). In contrast, only 1 gene was differentially expressed in mucosal biopsy specimens obtained from dogs with severe CE that did not have PLE versus those obtained from healthy control dogs.

Gene expression determined via QRT-PCR assay—Quantitative reverse transcriptase PCR assay was performed to determine expression of select genes for which significantly different expression was detected between healthy control dogs and dogs with CE on the basis of microchip array analysis. Six genes of interest were selected on the basis that different levels of expression between groups of dogs had been detected via microchip array analysis (acidic [leucine-rich] nuclear phosphoprotein 32 family member A and fibrillarin), genes encoded for cell-surface molecules (granzyme B, caspase 3, and chloride channel 3), or the genes encoded for molecules with a putative role in mediating intestinal inflammation (granzyme B, caspase 3, and matrix metalloproteinase 1). Results of QRT-PCR assay for these genes were consistent with results of microchip array analysis (Figure 2).

Gene expression in dogs with CE versus that in humans with IBD—Twenty-one of the 25 genes with the greatest difference in expression between dogs with CE and healthy control dogs are upregulated or downregulated in humans with IBD (Table 3). Four of the genes with significantly different expression between dogs with CE and healthy control dogs (CXCL13, cell division cycle associated protein 3, caspase 3, and tachykinin precursor 1) are not differentially expressed

in humans with CD or UC. Of the genes that are upregulated in humans with IBD, CD96 molecule, interleukin-8, tumor necrosis factor, peroxisome proliferator-activated receptor γ , RNase A family 1, S100 calcium-binding protein G, caspase 3, granzyme B, matrix metalloproteinase 1, matrix metalloproteinase 3, matrix metalloproteinase 13, and solute carrier family 40 member 1 were upregulated in dogs with CE. Conversely, 9 of the 21 genes upregulated in humans with IBD were downregulated in dogs with CE.

Discussion

Dogs that are genetically predisposed to the most prevalent forms of CE, including idiopathic IBD, have aberrant immune responses and dysbiotic microbiota.^{1,3,5,7,22} Chronic enteropathies in dogs are associated with abnormalities in mucosal immune cell populations and cytokine expression, activation of NF- κ B, and histopathologically detectable intestinal inflammation.^{1,4,23–27} Results of a recent study²² indicate intestinal inflammation is associated with a shift in the most prevalent intestinal microbes from gram-positive Firmicutes (eg, order Clostridiales) to gram-negative bacteria (predominantly Proteobacteria [including Enterobacteriaceae]). Granulomatous colitis in Boxers is associated with invasive *Escherichia coli* that are similar to mucosally adherent and invasive *E coli* associated with intestinal inflammation in humans.²⁸ Furthermore, the findings of other investigators that mucosal expression of Toll-like receptor-2, -4, and -9 is upregulated in various breeds of dogs²⁹ and that German Shepherd Dogs with CE have polymorphisms of Toll-like receptor-5⁵ indicate that interaction between intestinal microbes and the innate immune system is important. Thus, it was our intent to evaluate intestinal mucosal gene expression patterns in dogs with CE to identify genes that might be involved in CE disease mechanisms and to compare results with those reported for humans with IBD.

Results of microchip array analysis in the present study indicated that expression of > 1,870 genes was significantly different between healthy control dogs and dogs with CE. The functional importance of these differences in gene expression was determined by use of annotation-based GO databases. Results of initial GO analysis indicated that 42 biological process terms, 30 cellular components, and 18 molecular function terms were significantly overrepresented (false discovery rate $\leq 1\%$) among the genes determined to have different expression between dogs with CE and healthy control dogs (data not shown). Bioinformatics resources^p were used in addition to this computational procedure to determine biologically important differences between gene expression in dogs with CE versus that in healthy control dogs.^{13,30}

Results indicated that a majority (1,582 of 1,875 [84%]) of differentially expressed genes were downregulated (rather than upregulated) in dogs with CE in the present study. Neurotensin was the most highly downregulated gene in these dogs. Upregulation of neurotensin is associated with acute inflammation in humans with IBD and repair of injured epithelium (via

stimulation of expression of epithelial growth factor receptor) in rats and mice with experimentally induced colitis.^{31,32} Glucagon and cell division cycle associated A3 protein have roles in cellular replication via different mechanisms. The cell division cycle-associated A3 gene in humans encodes a protein that is required for controlled entry of cells into mitosis; this protein has not been identified in dogs.³³ Glucagon-like peptide-2, a product of posttranslational processing of proglucagon in the intestine, has intestinal growth-promoting properties.³⁴ Results of another study³⁵ indicate that glucagon-like peptide-2 attenuates intestinal inflammation in rodents with experimentally induced IBD, suggesting a potential role for this peptide in treatment of IBD in humans. It is possible that dogs with CE in the present study may have had low intestinal mucosal concentrations of glucagon-like peptide-2, which may have contributed to impaired intestinal protection against and repair following mucosal damage.

Fatty acids have an important role in inflammatory processes via nutritional and protective effects on enterocytes.³⁶ Results of another study⁹ indicate that genes involved in expression of fatty acid transport, fatty acid binding, fatty acid acetylation, and fatty acid synthesis proteins are downregulated in ileal and colonic tissues of humans with CD and UC. Findings of the present study that expression of fatty acid-binding protein 6 and fatty acid synthase were 13-fold and 3-fold as low, respectively, in intestinal mucosal biopsy specimens obtained from dogs with CE as in biopsy specimens obtained from healthy control dogs indicated dogs with CE had alterations in fatty acid metabolism similar to those detected in humans with IBD.

Intestinal epithelial barrier function is impaired in animals with IBD.^{13,37,38} Expression of several genes associated with maintenance of the intestinal epithelial barrier, including metallothionein 1E, metallothionein 2A, and claudin 8, was downregulated in dogs with CE in the present study. Metallothioneins play a central role in zinc homeostasis, modulate activation of the nuclear transcription factor NF- κ B, and serve as antioxidants to reduce intestinal inflammation.^{39,40} Claudins are a family of proteins that contribute to intestinal barrier function as components of tight junction strands between intestinal epithelial cells.⁴¹ Changes in expression and distribution of claudin 8 have been detected in humans with active CD and lead to altered tight junction structure and barrier dysfunction.⁴² Results of other studies^{43,44} indicate expression of matrix metalloproteinases in intestinal mucosa of IBD patients is a marker of disease activity, and these enzymes may be targets for treatment. Matrix metalloproteinases are involved in tissue remodeling, angiogenesis, and leukocyte extravasation in inflamed intestinal mucosa in humans with CD or UC.⁴⁵ Many of the matrix metalloproteinases investigated in those studies were also upregulated in dogs with CE in the present study, in particular matrix metalloproteinases 1, 3, and 13.

Numerous immune and inflammatory response genes were upregulated in dogs with CE in the present study. Members of the S100 calcium-binding protein family of genes are overexpressed in humans with IBD,⁸ and higher expression of S100 calcium-binding

protein G, S100 calcium-binding protein A2, and S100 calcium-binding protein A10 was detected in dogs with CE versus healthy control dogs in the present study. Moreover, expression of carcinoembryonic antigen-related cell adhesion molecule 18 (a marker of neutrophil activation and regulator of T-cell function) was also increased in dogs with CE, which suggested that there was infiltration of neutrophils in intestinal lesions, possibly in response to intestinal microbes.⁴⁶ The peroxisome proliferator-activated receptor γ gene encodes for a nuclear hormone receptor that regulates intestinal inflammation via antagonism of the transcription factor NF- κ B.⁴⁷ Peroxisome proliferator-activated receptor γ is highly expressed in a variety of cell types involved in intestinal inflammation, including monocytes, macrophages, T cells, dendritic cells, and intestinal epithelial cells. Increased epithelial expression of this protein is associated with attenuation of inflammatory intestinal lesions in experimentally induced IBD in mice.⁴⁸ It is possible that upregulated expression of this gene in dogs with CE in the present study was attributable to a compensatory response to chronic mucosal inflammation.

The finding of the present study that the caspase 3 and granzyme B genes were overexpressed in dogs with CE indicated that apoptotic mechanisms may have been involved in pathogenesis of the disease in those dogs. Resistance of T cells against apoptosis may contribute to persistent intestinal inflammation in humans with IBD.⁴⁹ Granzyme B is a serine protease that is an important mediator of apoptosis of cells targeted by natural killer cells and cytotoxic CD8⁺ lymphocytes. The primary apoptotic mechanism of action of granzymes is perforin-mediated cytotoxicity.⁵⁰ Further investigation of the mechanisms controlling mucosal lymphocyte apoptosis in dogs with CE is warranted.

Several organic cation transporter genes, including solute carrier family 40 member 1 and solute carrier family 2, member 2 (SLC2A2) were overexpressed in dogs with CE in the present study. Solute carrier membrane transport proteins are diverse, are expressed in most mammalian cells, and transport charged and uncharged organic molecules and inorganic ions. Solute carrier family 40 member 1 is involved in basolateral iron transport, and members of solute carrier family 2 SLC2, which includes 14 proteins, are facilitated glucose transporters.⁵¹ Other organic cation transporter genes (solute carrier family 22, member 4 [SLC22A4], also known as organic cation transporter gene 1 [OCTN1]; and solute carrier family 22, member 5 [SLC22A5], also known as organic cation transporter gene 2 [OCTN2]) are expressed in epithelial cells and have been implicated in CD.⁵²

The present study had some limitations. Gene expression was assessed in mucosal biopsy specimens obtained at only 1 time from each dog; results provided evidence that expression of genes in dogs with CE was different from that in healthy control dogs only at the time CE was diagnosed. Results of future studies in which mucosal gene expression is determined at multiple times after diagnosis of CE in dogs may better elucidate the importance of specific patterns of gene expression in onset and progression of the disease. Also,

several of the significant differences in expression of genes between dogs with CE and healthy control dogs in the present study may be attributable to secondary, rather than primary, abnormalities of gene expression related to pathogenesis of CE. We chose to include a group of control dogs with no signs of gastroenteritis that were younger than the dogs with CE. The strict inclusion criteria for control dogs in the present study (dogs that were free of disease and had unremarkable results of diagnostic tests) resulted in inclusion of a small number of such dogs. However, inclusion of healthy control dogs of mixed breeds was considered preferable to inclusion of age-matched Beagles bred for use in research; such Beagles sometimes have evidence of intestinal disease.⁵³ Therefore, it is possible that some of the differences in gene expression were attributable to differences in age between the 2 groups of dogs. Nevertheless, the tissue sampling and microchip array analysis methods used in the present study yielded results that indicated patterns of gene expression in dogs with CE were broadly similar to those in humans with IBD, including expression of genes regulating immune and inflammatory responses, metabolism, cell proliferation, and epithelial structure and function.^{8,54-62}

Results of the present study indicated that the expression of multiple genes regulating mucosal inflammation was markedly altered in dogs with CE, compared with that in healthy control dogs. The differential expression of several genes (fatty acid-binding protein 6, metallothionein 1E, claudin 8, matrix metalloproteinase 1, peroxisome proliferator-activated receptor γ , S100 calcium-binding protein G, and solute carrier family 40 member 1) between groups of dogs in the present study indicated that the molecular pathogenesis of CE in dogs may be similar to that in chronic immune-mediated intestinal inflammation in humans with IBD. Future experiments that include dogs with naturally developing IBD may be warranted because genomic sequences of dogs and humans are highly similar⁶³ and longitudinal studies (eg, clinical trials) might be easily conducted.

- a. RNAlater, Qiagen, Germantown, Md.
- b. RNeasy total RNA mini kit, Qiagen, Germantown, Md.
- c. Agilent Bioanalyzer with 6000 Nano LabChip, Agilent Technologies Inc, Santa Clara, Calif.
- d. RT2 First Strand, SA Biosciences, Qiagen, Germantown, Md.
- e. Canine 2.0 Array Platform, Affymetrix, Santa Clara, Calif.
- f. GeneChip Expression 3'-Amplification Reagents, Affymetrix, Santa Clara, Calif.
- g. GeneChip Fluidics Station 450, Affymetrix, Santa Clara, Calif.
- h. GeneChip Scanner 3000 7G, Affymetrix, Santa Clara, Calif.
- i. BROADD1, Broad Institute, Cambridge, Mass.
- j. European Bioinformatics Institute, Hinxton, Cambridgeshire, England. Available at: www.ebi.ac.uk/Information/. Accessed May 9, 2005.
- k. Primer Express Software, version 2.0, Applied Biosystems, Carlsbad, Calif.
- l. Platinum SYBR Green qPCR SuperMix-UDG, Invitrogen, Carlsbad, Calif.
- m. Excel, Microsoft Corp, Redmond, Wash.
- n. ROX, Affymetrix, Santa Clara, Calif.
- o. GeneAmp 5700 Sequence Detection System, Applied Biosystems, Carlsbad, Calif.
- p. Affymetrix, Santa Clara, Calif.
- q. Database for Annotation, Visualization and Integrated Discovery, version 6.7 [database online]. National Institute of Allergy

and Infectious Diseases, Bethesda, Md. Available at: david.abcc.ncifcrf.gov. Accessed Jun 15, 2008.

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Appendix appears on the next page

Appendix

Primer sequences used for QRT-PCR amplification of mRNA of various genes in intestinal mucosal biopsy samples endoscopically obtained from 18 dogs with CE and 6 healthy control dogs.

Gene	Primer	Probe sequence (5' to 3')	GenBank accession No.
Granzyme B	Forward	TGT TAX TGC TGC TTG AGA GGA AGG	NC_006590
	Reverse	ACC TGC GTA TCT TGG CCT CAA TCT	
Glucuronidase β	Forward	ATG CTG GTC CAG AGC TAC AGC AAT	NC_006588
	Reverse	ATG GGT GAT TCT TAT CCC GAC GCA	
ANP 32 family member A	Forward	AAC AGG ACG CCC TCT GAT GTG AAA	NC_006612
	Reverse	TGA GGT GAG GCC TAC GTT GAT TGT	
Chloride channel 3	Forward	AGA AGG TCC TGG CTC GTA CAT CAT	NC_006607
	Reverse	CCA GGG AAA CAG CAA GAA AGG CAA	
MMP13	Forward	GAC AGA TTC TTC TGG CGA TTG CAT CC	NC_006587
	Reverse	TAT GCA GCA TCG ATA CGG TTG GGA	
Caspase 3	Forward	ATT ATT CAG GCC TGC CGA GGT ACA	NC_006598
	Reverse	TAC AAG AAG TCC GCT TCG ACT GGT	
Fibrillarin	Forward	TGC TCA TAG CAA TGG TGG ACG TGA	NC_006583
	Reverse	TAC GAA GGA AGG TGT GGG CAT TCA	