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P-glycoprotein Expression in Lamina Propria Lymphocytes of Duodenal Biopsy Samples in Dogs with Chronic Idiopathic Enteropathies

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Summary

P-glycoprotein (p-gp) is a transmembrane protein functioning as a drug-efflux pump in the intestinal epithelium. Human patients with inflammatory bowel disease (IBD) who fail to respond to treatment with steroids express high levels of p-gp in lamina propria lymphocytes. The purpose of this study was to investigate p-gp expression in duodenal biopsy samples of dogs with chronic enteropathies and to evaluate the expression of p-gp after treatment with a known inducer of p-gp (prednisolone). Duodenal biopsy samples from 48 dogs were evaluated immunohistochemically with the mouse monoclonal antibody C219 for expression of p-gp in lamina propria lymphocytes. Biopsy samples were available from 15 dogs after treatment with prednisolone and 16 dogs after dietary therapy alone ("elimination diet"). Treatment with prednisolone resulted in an increase in p-gp expression ($P=0.005$). In contrast, dietary treatment alone produced no significant change in p-gp expression ($P=0.59$). A low p-gp score before initiation of steroid treatment was significantly associated with a positive response to treatment ($P=0.01$). These results indicate that lamina propria lymphocyte expression of p-gp is upregulated after prednisolone treatment in dogs with IBD, and that mucosal expression of p-gp may be of volue in predicting the response to therapy.

Keywords: chronic enteropathies; corticosteroids; dog; p-glycoprotein

Introduction

Idiopathic inflammatory bowel disease (IBD) is the most common form of chronic intestinal disease in dogs (Hall and Simpson, 2000; Jergens and Willard, 2000). The diagnosis is based on clinical signs of chronic diarrhoea, vomiting and weight loss, combined with histological evidence of inflammatory infiltration of the intestinal mucosa. Treatment commonly consists of immunosuppressive doses of corticosteroids, to reduce the intestinal mucosal inflammation and bring about clinical remission (Hall and Simpson, 2000); some dogs, however, show no response, presenting the veterinarian with a dilemma. In human IBD patients, it is helpful to predict failure to respond to steroid treatment, which is observed in 20–30% of cases (Faubion *et al.*, 2001a,b), as such failure often leads to surgical intervention.

The anti-inflammatory effect of steroids in IBD is thought to be due to their action on T cells that infiltrate the mucosa. Steroids bind to intracellular receptors in susceptible T cells (Farrell and Kelleher, 2003). The ligand-receptor complex is then transported to the nucleus, where it regulates the transcription of target molecules, such as inhibitor kappa B alpha, which binds to, and inhibits, nuclear factor kappa B by sequestering it

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in the cytoplasm (Scheinman *et al.*, 1995). Nuclear factor kappa B is a potent anti-apoptotic and pro-inflammatory transcription factor, which is "upregulated" in human patients with IBD (Wild *et al.*, 2003). Steroid transport into the cell is thought to be a passive process, but extrusion from the cytoplasm depends on active efflux through cell-membrane-associated "pumps". P-glycoprotein (p-gp) is one of the main drug efflux pumps expressed in membranes of human lymphocytes, intestinal epithelial cells, biliary duct cells, proximal renal tubules, adrenal glands and choroid plexus epithelial cells in the brain (Thiebaut *et al.*, 1987). Numerous substrates for p-gp have been identified, one of the best known being glucocorticoids. In normal human intestinal epithelial cells, p-gp expression is slight in the duodenum and jejunum and more prominent in the ileum and colon (Mouly and Paine, 2003). In normal dogs, p-gp is expressed in high concentrations in the kidney, testes, liver and brain, and to a variable degree in peripheral blood leucocytes (Conrad *et al.*, 2001). Expression in normal epithelial cells of the canine duodenum is minimal, and only slightly greater in colonic epithelial cells (Conrad *et al.*, 2001). High expression of p-gp in T cells of human patients suffering from rheumatoid arthritis, systemic lupus erythematosus, drug-resistant epilepsy and renal graft rejection is associated with poor response to treatment (Zanker *et al.*, 1995; Maillefert *et al.*, 1996; Montano *et al.*, 1996; Diaz-Borjon *et al.*, 2000; Lazarowski *et al.*, 2004). Farrell *et al.* (2000) showed that human IBD patients who responded to steroid therapy differed from those who did not in expressing less p-gp in their peripheral T lymphocytes and intra-epithelial lymphatic cells. This suggests that glucocorticoids are effectively pumped out of the cells in patients expressing high amounts of p-gp on the surface of infiltrating lymphocytes, leading to low intracellular concentrations. This may result in an increased concentration of inflammatory cytokines in the intestine and, ultimately, reduced clinical efficacy of treatment.

The cellular infiltrate in canine chronic idiopathic enteropathies consists mainly of lymphocytes and plasma cells in the lamina propria (Jergens *et al.*, 1992). The mechanism of action of steroids in the treatment of canine IBD is thought to be similar to that in human IBD. So far, however, the mechanisms of glucocorticoid resistance in canine IBD have not been elucidated.

The purpose of this study was (1) to investigate p-gp expression in lymphocytes infiltrating the lamina propria in the duodenum of dogs with IBD, (2) to compare the degree of expression in affected dogs receiving high doses of corticosteroids with that in animals responding to treatment with a commercial "elimination diet", and (3) to relate expression to the clinical response to treatment. Our hypothesis was that p-gp expression in lamina propria lymphocytes in dogs with chronic enteropathies would be upregulated after treatment with prednisolone, and that dogs with high p-gp expression would respond poorly to treatment.

Materials and Methods

Selection of Cases and Histopathology

Eighty duodenal biopsy samples from 48 dogs with chronic idiopathic enteropathies of more than 6 weeks' duration were evaluated. The severity of clinical disease was assessed on the basis of the canine IBD activity index (CIBDAI), as proposed by Jergens *et al.* (2003); thus, a score of 0–3 indicated clinically insignificant disease, a score of 4–5 indicated mild IBD, a score of 6–8 indicated moderate IBD, and a score of ≥ 9 indicated severe clinical IBD. Clinical assessment was complemented by laboratory tests, which included a complete blood count, chemistry profile, urinalysis, parasitological and bacteriological examination of faeces, serum trypsin-like immunoreactivity (TLI), vitamin B_{12} and folate measurements, abdominal ultrasound, and endoscopy with intestinal biopsy sampling to exclude any other causes of diarrhoea. None of the dogs included in the study had previously been treated with steroids. After endoscopic examination and biopsy, all dogs received treatment for one week with an elimination diet, i.e., a diet containing a protein source never fed before (Hall and Simpson, 2000). If no clinical response was seen, immunosuppressive doses of prednisolone were added to the treatment regimen (1 mg/kg orally twice daily for 10 days, then 0.5 mg/kg orally twice daily for 10 days, then 0.5 mg/kg orally once daily for 10 days, then 0.5 mg/kg orally every other day for 10 days). If the dogs responded well to the initial 7-day treatment with elimination diet alone, it was continued for a total of 10 weeks. A further examination by endoscopy and biopsy was performed in 15 dogs responding solely to elimination diet, as well as in 16 dogs requiring additional steroid treatment. Response to treatment was defined as a reduction of CIBDAI to a score of 0 to 1 after treatment; failure to respond was defined as minimal if any reduction in CIBDAI. Histological assessment and scoring of the severity of infiltration in the lamina

propria was performed as proposed by Jergens *et al.* (1992).

Immunohistochemical Technique

Immunolabelling was performed by the streptavidin-biotin peroxidase technique with mouse anti-human p-gp monoclonal antibody (C219; DAKO, High Wycombe, UK), diluted 1 in 20 in phosphate-buffered saline (PBS; 0.01 M, pH 7.4), on formalin-fixed biopsy samples. After incubation with proteinase K (DAKO) 20 μg/ml for antigen retrieval, the sections were incubated for 1 h at 37 °C in a humid chamber with the diluted primary antibody. The sections were then washed in PBS and incubated first with the secondary antibody (anti-mouse IgG conjugated with biotin; DAKO) for 1 h at 37 °C in a humid chamber and then with streptavidin-peroxidase complex (DAKO) for 30 min at room temperature. After incubation with DAB chromogenic substrate solution (diaminobenzidine 0.02% and H_2O_2 0.001% in PBS; DAKO) for 5 min, sections were rinsed with PBS and running tap water, counterstained with haematoxylin, and mounted with permanent mounting medium (DAKO). Normal canine liver and canine high-grade lymphoma (positive for p-gp expression) were used as positive controls (Bergman *et al.*, 1996). As a negative control for the immunolabelling procedures, 10% normal mouse serum was used instead of the primary antibody. Surgical duodenal biopsy samples from 10 clinically healthy beagles (humanely destroyed in an unrelated study) served as controls for p-gp expression in normal intestine.

Examination of Immunohistochemistry Sections

Slides with sections from biopsy material were coded and examined with a Leica DRMB microscope and Panasonic F15 DCC camera by a single investigator (KA) without knowledge of their origin. Total lymphocytes and lymphocytes labelled with C219 were counted per 10 000 μm^2 of lamina propria in which epithelial and vascular structures had been excluded. A score was applied to each biopsy sample according to a scoring system adapted from German *et al.* (1998, 1999). From each biopsy sample, 20 areas of the lamina propria were selected, 10 from villous areas and 10 from crypt areas. A count of positive cells per 10 000 μm^2 for each of the 20 areas was then made and the mean value (p-gp score) calculated (Fig. 1). If no positive lymphocytes were seen in any of the 20 areas examined, the p-gp score was 1; for a mean of 1–4 positive cells, the score was 2; for a mean of 5–10, the score was 3; and for a mean of >10 positive cells, the score was 4. C219 has been previously found to label skeletal and heart muscle cells (Cordon-Cardo *et al.*, 1990), but the chance of overestimation of p-gp expression was deemed to be minimal, as care was taken to count only positive cells with lymphoid morphology.

Statistics

A statistical software package was used for all calculations (NCSS Statistical Software, Kaysville, Utah, USA). Comparison of C219 scores between the group of dogs treated with steroids versus the dogs treated with diet alone was made by the non-parametric Mann-Whitney U test (on score ranks) or Chi^2 test (on score category counts). The Mann-Whitney U test or Wilcoxon signed rank test was applied for difference in medians between the steroid-treated group before and after treatment and the food-responsive group before and after treatment (paired test). The statistical significance was set at $P<0.05$.

Results

Comparison of Dogs with Diet-responsive vs Steroid-treated Chronic Enteropathy

The grouping of dogs was made retrospectively after they had all been treated with elimination diet for one week. Of 24 dogs needing additional treatment with steroids, 16 responded.

The group of 24 dogs with diet-responsive enteropathy consisted of 15 males, of which three were neutered, and nine females, of which five were neutered. These animals were 0.5–10 years old (mean 2.64 years, median 1.7 years) and belonged to the following breeds: mixed ($n=5$), German shepherd dog (3), golden retriever (2), Bernese mountain dog (2), dachshund (2), West Highland white terrier (1), Border collie (1), Cairn terrier (1), Chihuahua (1), Great Dane (1), Landseer (1), Malinois (1), whippet (1), Dobermann pinscher (1) and Leonberger (1). Dogs (24) selected for steroid therapy included 16 males, of which three were neutered, and eight females, of which seven were neutered. These animals were 0.5–10 years old (mean 5.84, median 4.2 years), and belonged to the following breeds: Yorkshire terrier ($n=4$), mixed (3), dachshund (3), German shepherd dog (2), golden retriever (2), Shar pei (2), Rottweiler (2), Labrador retriever (1), boxer (1), greyhound (1), miniature schnauzer (1), miniature poodle (1) and mastiff (1). There were no statistically significant differences in the sex distribution between the two

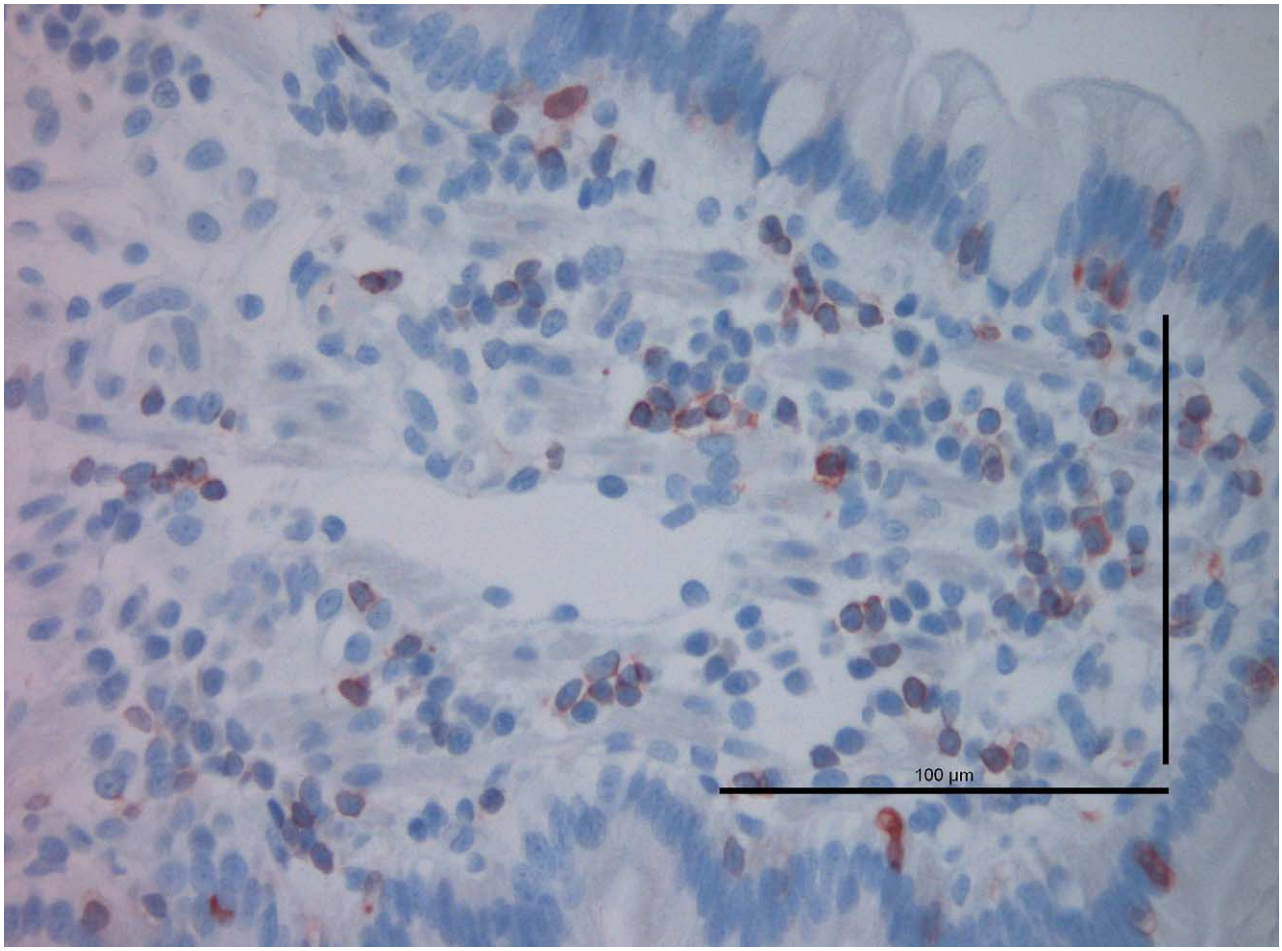

Fig. 1. Section of duodenum from a dog with chronic idiopatic enteropathy, demonstrating infiltration of the duodenal lamina propria with p-glycoprotein (p-gp)-positive lymphocytes. Streptavidin-biotin immunoperoxidase technique.

groups. There were significantly more young dogs (<2 years of age) in the diet responsive group than in the steroid group (mostly aged between 3-7 years) (Chi^2 test, $P=0.0002$). The clinical scoring was also significantly higher in the group of dogs that had to be treated with steroids (Chi^2 test, $P=0.000001$).

P-glycoprotein Expression in Normal Canine Duodenum

No expression of p-gp was found either in epithelial cells or in the rare lamina propria lymphatic cells of intestinal biopsy samples from 10 clinically healthy beagles.

Histopathological Scoring of Duodenal Biopsy Samples from Dogs with Diet-responsive or Steroid-responsive Enteropathy, and Total Counts of Lymphocytes in the Lamina Propria

Comparisons made between the two groups before the start of steroid therapy revealed no significant difference in terms of (1) histopathological score (Chi^2, $P=0.09$), or (2) total lymphocyte count (median values 154 and 166 cells/10 000 μm^2, respectively; Mann-Whitney U test, $P=0.06$).

P-glycoprotein Expression in Dogs with Diet-responsive or Steroid-responsive Enteropathy: Counts Before and After Treatment

Scoring of p-gp expression did not change significantly in the dietary treatment group (Wilcoxon signed rank test, $P=0.59$) (Fig. 2). In contrast, p-gp expression increased significantly as a result of therapy in the steroid-treatment group (Wilcoxon signed rank test, $P=0.005$) (Fig. 2).

Association of P-glycoprotein Expression with Response to Steroid Treatment

A statistically significant association between a positive response to treatment and a low p-gp scoring was found when dogs of the

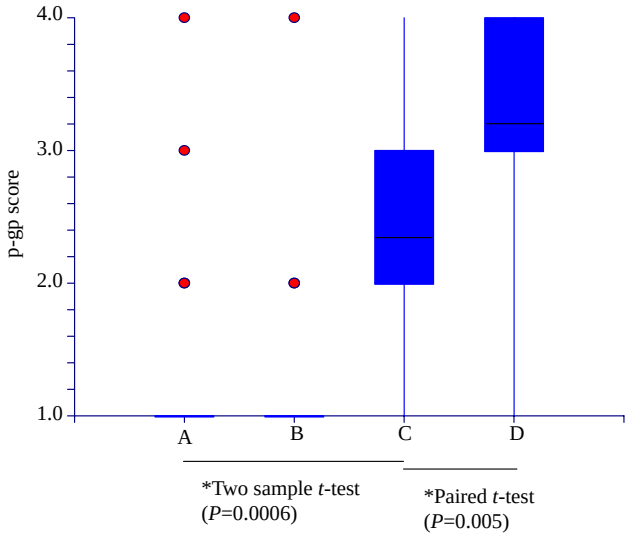

Fig. 2. Boxplots representing p-gp scores in duodenal biopsy samples from dogs with chronic enteropathies. An indirect immunoperoxidase technique was used to label sections from 15 dogs before (A) and after (B) 10 weeks of dietary treatment alone, and from 16 dogs before (C) and 10 weeks after (D) treatment with diet and steroids.Significant differences (*) were detected between p-gp scores before and after steroid treatment (C vs D; paired *t*-test, Wilcoxon signed rank test, $P=0.005$), and between dogs treated with either diet or steroids (A vs C; two-sample *t*-test, Wilcoxon signed rank test, $P=0.0006$). Bars in boxes represent medians.

steroid-treatment group were evaluated for p-gp expression before initiation of treatment. Of 16 dogs that responded to treatment, 14 gave p-gp scores of 1 or 2. In contrast, of eight non-responding animals, seven initially gave p-gp scores of 3 or 4 (Chi^2, $P=0.0001$).

Discussion

Idiopathic chronic enteropathies are usually treated initially by feeding an elimination diet (Hall and Simpson, 2000). If the animal responds, no further intervention is necessary. If the dietary approach is unsuccessful, the next line of treatment is steroid therapy, usually beginning at an immunosuppressive dosage which is reduced over a period of several weeks or months (Jergens *et al.*, 1992). Some dogs, however, will not respond to steroid-treatment and these pose a significant challenge to the veterinarian. A method for identifying probable non-responders before starting treatment would be helpful. In several human diseases, including IBD, p-gp has been used successfully for such a purpose (Farrell *et al.*, 2000). For this reason, the present study of canine IBD was designed to investigate expression of p-gp in duodenal biopsy samples from dogs responding to (1) dietary treatment alone, or (2) prednisolone treatment. The latter treatment was shown to be associated with significantly higher p-pg expression. In addition, a statistically significant association between a positive response to treatment and a low p-gp scoring was found when dogs of the steroid-treatment group were scored before initiation of treatment.

The investigations were concentrated on p-gp expression in duodenal lamina propria lymphocytes, and did not include the colonic mucosa. Although high p-gp expression is usually observed in canine colonic epithelium, it cannot be excluded that lymphocytes infiltrating the colonic mucosa may contribute to treatment failure. However, the colonic epithelium does not contribute significantly to the absorption of nutrients or drugs, and it may therefore not be as relevant as the duodenum with regard to steroid absorption and action in the mucosa. In addition, all dogs included in the study appeared clinically to be suffering from a mainly small intestinal disease. It is likely that the main infiltration site of the mucosa is of particular importance with regard to drug sensitivity.

The numbers of dogs included in each treatment group were the same ($n=24$), but the median age in the dietary treatment group (1.7 years) was significantly lower than in the steroid-treatment group (4.2 years). In addition, the clinical scoring was lower in the dietary treatment group. The younger age in the dietary treatment group points towards possible food intolerance or food allergy in these animals, as such diseases occur more frequently in young dogs (Jergens *et al.*, 1992; Guilford, 1994; Paterson, 1995). It is also possible

that dogs with severe disease at the time of first diagnosis were particularly likely to be switched to steroid treatment after one week of diet treatment, as further clinical deterioration could not be permitted. However, neither the histological scoring of the biopsy samples nor the total lymphocyte counts per 10000 μm^2 differed between the two treatment groups. In addition, the total lymphocyte counts observed in the duodenal biopsy samples were comparable with those of other dogs with chronic enteropathies (German *et al.*, 2001). It has been suggested that awareness of the type and degree of histological infiltrates in canine IBD may not be as helpful as in human IBD, in which clinical scores are closely related to histological grading (German *et al.*, 2001, Willard *et al.*, 2001). Clearly, other parameters are necessary for diagnosis, grading of disease severity, and assessment of therapeutic response in dogs with chronic enteropathies.

P-glycoprotein expression was already higher in the steroid-treated group before the animals received any steroids. This suggests that expression of this cell-surface protein in the lymphocytes occurred independently of any drugs. Therefore, p-gp expression in the lymphocytes of the lamina propria may provide the clinician with a tool to identify cases that need more intensive treatment, such as steroids in addition to dietary treatment. After treatment, p-gp expression was significantly higher in the steroid-responsive group ($P=0.005$); no difference in the scores was observed, however, in the dietary treatment group. This finding accords with a study on dogs with lymphoma, which showed that upregulation of the multiple drug resistance (MDR) protein could occur in some canine lymphoma cells after treatment with steroids (Bergman *et al.*, 1996).

In the present study it was found that, in dogs with steroid-responsive chronic enteropathy, a low p-gp score before starting treatment was significantly associated with a good therapeutic response. It would seem, therefore, that p-gp expression is capable of serving as a marker for identifying dogs unlikely to respond to steroid treatment. In the future, the diagnostic approach in dogs with chronic intestinal disease may be based on a clinical examination including gastrointestinal endoscopy, followed by treatment with an elimination diet. In the absence of a response to dietary treatment alone, immunolabelling for p-gp in intestinal biopsy samples could be recommended to predict the response to treatment with prednisolone. Dogs with high p-gp scores before treatment might need to be treated more aggressively, e.g., with drugs such as azathioprine or cyclosporine, in addition to steroids.

On the basis of the results of this study, it is speculated that high expression of the drug efflux pump p-gp in lymphocytes infiltrating the small intestinal mucosa may render these cells less responsive to steroid treatment, leading to increased local concentrations of pro-inflammatory cytokines and more severe clinical disease.

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